THE UNIVERSITY OF ALBERTA

METHEMOGLOBIN REDUCTION IN RABBIT ERYTHROCYTES

A THESIS

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by

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ABSTRACT

The erythrocyte maintains a delicate balance between two oxidation states of hemoglobin, such that the biologically active, but thermodynamically unstable, ferrous hemoglobin predominates. The enzymic mechanisms by which the ferrous hemoglobin is maintained in the reduced state are not understood.

Two metabolic pathways are available to supply the electrons required for methemoglobin reduction; the glycolytic pathway, using diphosphopyridine nucleotide, and the pentose phosphate pathway, which uses triphosphopyridine nucleotide. Although the literature contains evidence that reductase sequences utilizing both of the pyridine nucleotides are present in the erythrocyte, the number and nature of the methemoglobin reductase sequences are still unknown.

Our results indicate that there are two pyridine nucleotide specific reductases in the erythrocyte. One of the enzymes found was triphosphopyridine nucleotide specific. Its sensitivity to carbon monoxide suggests that it is a heme enzyme. A similar methemoglobin reductase has been previously isolated but this *in vitro* enzyme had an absolute requirement for methylene blue. This was not the case with our enzyme system. The second enzyme present in our system was diphosphopyridine nucleotide specific and required a thiol group for activity. Whether the thiol is associated with the enzyme or the MHb substrate is unknown. Some data concerning the reaction sequence of the latter enzyme are also presented.

It has been suggested that the dye reductase found in erythrocytes is a methemoglobin reductase. An assay for the erythrocyte dye reductase was developed and the enzyme was shown to be a sulfhydryl enzyme, but no conclusion was reached as to the true function of the dye reductase.

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LIST OF ABBREVIATIONS

Hemoglobin (Fe^{2+})	Hb
Methemoglobin (Fe^{3+})	MHb
Carboxyhemoglobin	Hb(CO)
Diphosphopyridine nucleotide	
oxidized form	DPN^+
reduced form	DPNH
Triphosphopyridine nucleotide	
oxidized form	TPN^+
reduced form	TPNH
Glutathione	GSH
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Tris (hydroxymethyl) aminomethane	Tris
p-chloromercuribenzoate	PCMB
Glyceraldehyde-3-P	triose-P
Ethylene-diamine-tetraacetic acid	EDTA

The policy of the Journal of Biological Chemistry was followed for chemical substances and for the units of measure.

Where used, mg % or mg per cent means mg per 100 ml.

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GENERAL INTRODUCTION

The mammalian erythrocyte possesses the physiological function of oxygen transport. It is performed in the red cell by hemoglobin (Hb). The hemoglobin molecule is an excellent example of the adaption of molecular structure to biological function. The effects of changing pO_2 and pH upon the Hb - O_2 equilibrium, cause a maximum release of O_2 to the tissues where it is needed and saturation of the Hb with O_2 in the lungs where the pO_2 is highest.

This molecular paragon has two oxidation states, known as hemoglobin (Fe^{2+}) and methemoglobin (Fe^{3+}). Hb is the biologically active form whereas methemoglobin (MHb) is the thermodynamically stable entity (1). In addition, Hb reacts chemically with O_2 to form MHb (2). Hence, at equilibrium in an open system at physiological pH considerable MHb will be present, which is to say that the equilibrium:

$$MHb \rightleftharpoons Hb + 4\,e^-$$

will be far to the left.

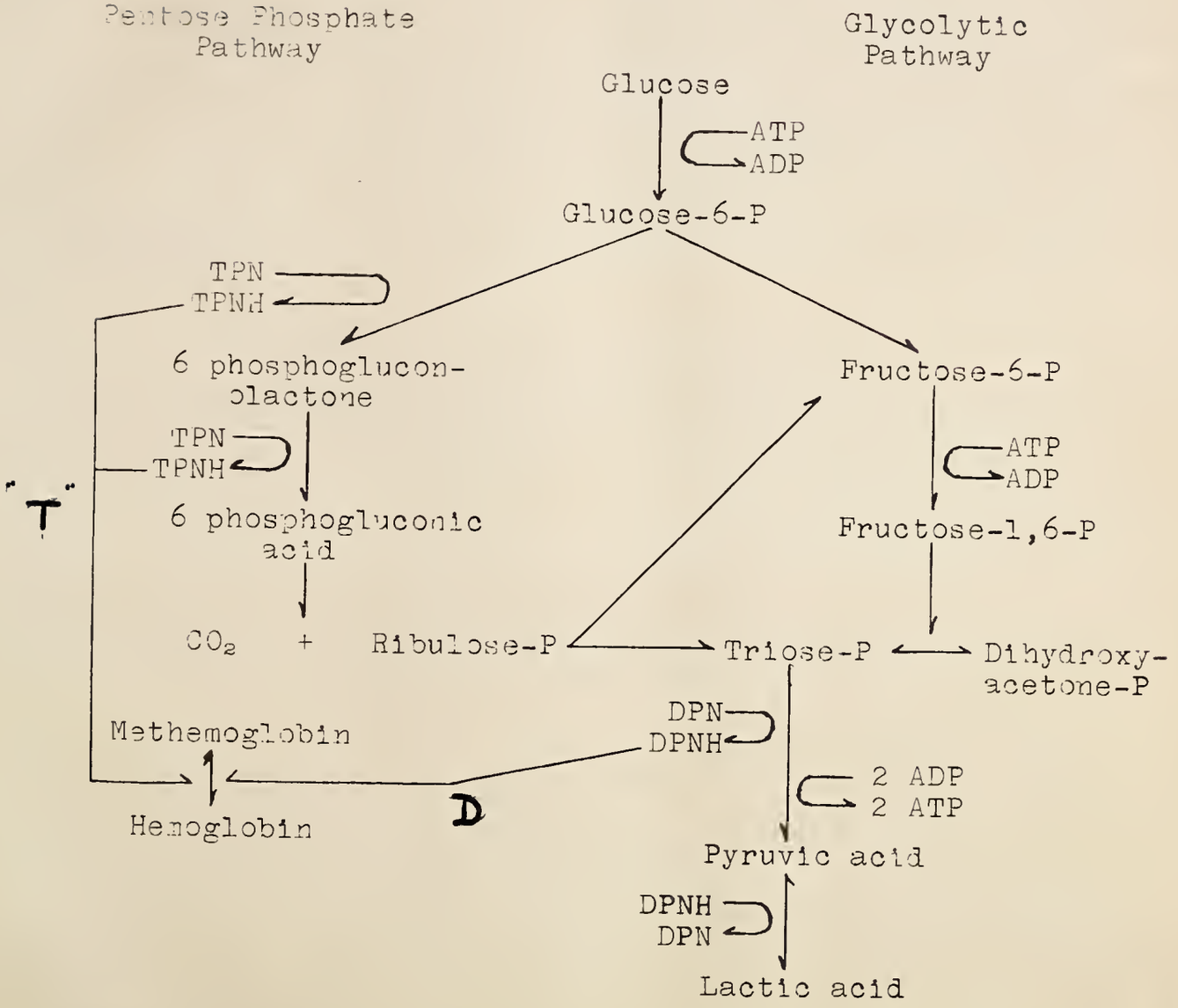
The low steady level of MHb (about 1%(3)) in the living cell indicates that some mechanism is present to counteract the effects of O_2, and to prevent the system from attaining chemical equilibrium. The maintenance of Hb against a thermodynamic gradient requires energy which in the erythrocyte comes from glucose metabolism. The literature contains many reports (4-9) of experiments in which methemoglobinemic erythrocytes were incubated with, and without, glucose. In all cases, only those cells incubated with glucose reduced MHb. Lactic acid may also be used as the substrate for MHb reduction, (4,8) but the main energy supply comes from glucose (5).

Glucose metabolism in the mature erythrocyte proceeds via both the glycolytic, and the pentose phosphate pathways (10). The majority is metabolized by the glycolytic sequence in the human erythrocyte. The erythrocytes of other species have not been studied, but in animals that have erythrocytes similar to those of the human (11), there is no reason to believe that the proportion of glucose metabolized by the two pathways is strikingly different. Rabbit erythrocytes fall in the same category as the human cell.

In fig.1, the glucose metabolism of the human erythrocyte is diagramatically represented. It will be noted that the two metabolic sequences require different pyridine nucleotides. The known electron transport sequences start with a pyridine nucleotide specific enzyme and, by analogy, the MHb reductase sequence should also be pyridine nucleotide specific. Therefore, if there were only one MHb reductase, only one pyridine nucleotide would be used, and only one metabolic sequence would be involved in MHb reduction. However, the relative contribution of the two pathways to the metabolism of the methemoglobinemic cell has not been determined. Except for the study by Murphy (10) in which normal cells were used, this phase of erythrocyte metabolism has been ignored. The result is that no investigator can say which metabolic pathway is used by the erythrocyte to supply the electrons for MHb reduction. Despite this fact, there has been a dispute in the literature for some time as to which pyridine nucleotide is used by the cell for MHb reduction. Some of the dogmatic statements that have been made are not valid in view of the fact that the metabolism has not been elucidated. There is evidence in the literature for both the system labelled "D"

Fig. 1

Glucose Metabolism in the Erythrocyte



This fig. was adapted from a paper by J. R. Murphy(10).

and that labelled "T" in fig. 1.*

In 1957, Heunnekins and co-workers (12) reported the isolation of a MHb reductase T. The enzyme was a heme protein and had an absolute requirement for methylene blue. Kiese (13) has isolated a similar enzyme. Originally, Kiese claimed that flavin was present in his enzyme, but communications with Heunnekins have indicated that further purification of his material has removed the flavin. Huisman and Meyering (14) have also isolated this heme enzyme during their work on Hb heterogeneity.

No isolation of a MHb reductase D has been reported. Scott and Griffith (15) attributed this to a sensitivity to organic solvents that is exhibited by the MHb reductase D. The most common method for the removal of Hb from enzyme preparations is a low temperature treatment with $CHCl_3$ and C_2H_5OH which would destroy the MHb reductase D. However, much indirect evidence for the presence of the enzyme has been presented.

The strongest indications of the presence of MHb reductase D come from metabolic experiments. Warburg and coworkers (4), Gibson (8), Wendel (16) and Jaffe (17) have reported that erythrocytes will utilize lactic acid as a substrate for MHb reduction. Lactic dehydrogenase oxidizes lactic acid to pyruvate with coincident reduction of DPN^+. The pyruvate produced could be quantitatively recovered (8) and a good correlation between the amounts of pyruvate produced and the MHb reduced was obtained. From these facts, it was concluded that DPNH is involved in MHb reduction.

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*The reductase systems are referred to throughout the remainder of the thesis as MHb reductase "T" or "D" according to the pyridine nucleotide involved.

Bodansky, Gutmann and Jandorff (18) isolated DPN^+ in crude form, reduced it, and used the resulting crude DPNH preparation as a substrate for MHb reduction in an hemolysate system. Their enzyme preparations were less active than the whole cell and the DPN^+ preparation probably contained TPN^+, but their work is the only recorded study in which DPNH was used as the substrate for MHb reduction. No reports of studies using TPNH under similar conditions have appeared.

Further evidence for the presence of a MHb reductase D comes from the study of various congenital methemoglobinemias, and the genetic defect of erythrocytes known as "primaquine sensitivity". In this latter condition, the metabolic lesion is a lowered activity of glucose-6-P dehydrogenase (19). The lesion effectively lowers the amount of TPNH available to the cell, as is evidenced by lowered GSH levels and high fragility. If TPNH were the only pyridine nucleotide involved in MHb reduction, cyanosis would be expected to be a maximum in these patients but the 25-30 per cent MHb levels found in these people (17) is not the 50-70 per cent common in congenital methemoglobinemias. This would indicate the presence of another MHb reductase that was not coupled to glucose-6-P dehydrogenase, such as a MHb reductase D.

The lesion is only a lowered glucose-6-P dehydrogenase activity and the effects noted could be the result of the cell existing with a lower than normal supply of TPNH. The evidence for MHb reductase D from this source is weak and must be considered only with more conclusive data.

The lesion in congenital methemoglobinemia may take one of two

forms. Pisciotta, Ebbe, and Hinz (20) reported a methemoglobinemia in which an abnormal Hb is present (MHb M disease). They postulated that the MHb M cannot be reduced to Hb M because of the incompatibility of Hb M and MHb reductase. The postulate continues that the MHb reductases in these patients are normal. From this, one would expect that if MHb A were supplied as the substrate, MHb reduction would occur normally. However, no experiments of this nature have been reported. If the postulates concerning MHb M disease are true, it would be interesting to compare the structures of MHb M and MHb A by Xray diffraction to determine the differences that cause the incompatibility. The knowledge of MHb M disease is limited as very few cases have been reported.

The second form of methemoglobinemia has greater significance in the study of MHb reduction. This second form is characterised by a lowered ability to reduce MHb in the erythrocyte. Cells with this lesion, have a normal glycolytic metabolism (8) and utilize lactic acid in vitro at about the same rate as do normal cells. Since MHb reduction does occur with lactic acid, by similar reasoning to that used previously, the use of this energy source implies the coincident reduction of DPN^+ by a DPN^+ coupled reductase.

Gibson (8), from studies on such erythrocytes, postulated that some diaphorase-like material was missing from the reductase sequence when this disease is present. Scott and Hoskins (21) reported that the methemoglobinemia found in Eskimos was similar, and their data indicate that the cells were low in both MHb reductase D and T. However, subsequently Scott and Griffith (15) described the lesion in these people as an absence of diaphorase. Because the erythrocyte is DPNH

specific, a slight contradiction is present in the two papers. Despite this, there is some evidence in their work for a DPNH linked MHb reductase. The work of Scott and Griffith contains the only experimental evidence that diaphorase and MHb reductase activity are properties of a single protein.

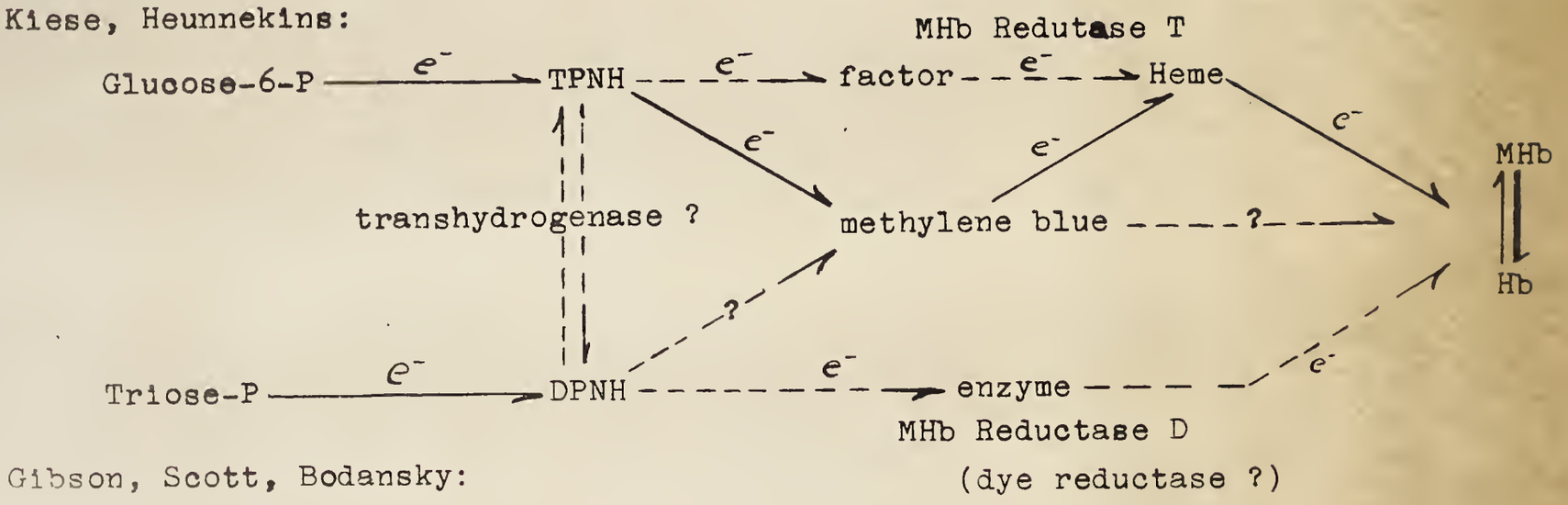
The idea that these two activities are properties of the same enzyme is not new. Gibson (8) postulated such a relationship, and the dye methylene blue has been intimately connected with MHb reduction for a long time (4). Heunnekins et al (12) and Kiese (13) both use dyestuffs in their studies of MHb reductase. The standard textbook treatment for cyanosis is a massive injection of methylene blue (22). Murphy (10) showed that methylene blue stimulated the pentose phosphate pathway and Gibson suggested that the dye produced a shift in the MHb reductase T sequence, that resulted in a new faster reduction chain. Other workers (12,16) concur with these ideas.

Heunnekins et al (12) used the dye, methylene blue, in their isolation of the MHb reductase T. The methylene blue stimulates the O_2 consumption of erythrocytes and the MHb reduction rate, but the exact reason why the dye does this is unknown. The stimulation of MHb reduction occurs also with ascorbic acid and Vitamin K_3 stimulates the O_2 uptake. A general correlation between the ability to stimulate MHb reduction and O_2 uptake would be a great help in the study of MHb reduction as the O_2 consumption is 7 times as fast as the MHb reduction (12). Some experiments were done to test this correlation.

Present concepts of MHb reduction are diagramatically summerized in fig. 2. Solid arrows indicate reactions that are accepted;

Fig. 2

MHb Reduction in the Erythrocyte



Solid lines indicate reactions that may be considered as proven present.

Broken lines indiacte postulated reactions that have some evidence to show their existence.

dotted arrows indicate postulated, possible or disputed reactions. From the data in the literature, both the D and T MHb reductases should be present in the erythrocyte. The possibility of a transhydrogenase in erythrocytes, has not been investigated and the reduction that occurs with one or the other pyridine nucleotide could be the result of a sequence such as:

$$\text{Substrate} \xrightarrow{e^-} \text{DPN}^+ \xrightarrow{e^-} \text{TPN}^+ \xrightarrow{e^-} \text{Enzyme} \xrightarrow{e^-} \text{MHb}$$

None of the previous data can be used in argument against such a scheme.

It was the purpose of the study reported in this thesis to reinvestigate the problem of MHb reduction and to establish the presence of both MHb reductase D and MHb reductase T in the rabbit erythrocyte, and if possible, to look into the mechanism of the reductase reactions. It was further proposed to establish assay procedures that could be used in a future isolation of the reductases.

Since the ultimate goal of the study was the isolation of the reductases, and because of the postulate of, and meager evidence for a connection between the methemoglobin reductases and erythrocyte dye reductase, it was decided to do a study with erythrocyte dye reductase paralleling that on the MHb reductases. The procedure developed for the dye reductase study could then be used to estimate the reductase activity of the various stages in the separation of the MHb reductases, to prove whether the two activities do, indeed, come from a single protein.

IN VITRO EFFECTS OF ANTI-CYANOTIC DRUGS

I. Introduction

Two chemicals, methylene blue and ascorbic acid, are commonly administered in the treatment of cyanosis (3,22). Presumably, these compounds either increase the activity of the pentose phosphate pathway, so increasing the MHb reduction rate (8,10) or react directly with MHb to produce the same effect.

In vitro, methylene blue stimulates the normally anaerobic metabolism of the erythrocyte to a very active pseudo-aerobic state (23). Vitamin K_3 also stimulates the aerobic metabolism of the erythrocyte (24). No data are available concerning the effect of ascorbic acid.

Several workers (6,8,12) have suggested that methylene blue caused a change in the metabolic pathways, producing a new sequence that is much more active than the normal process. This explanation of the action of methylene blue can be applied to its effect on either respiration, or on MHb reduction. A few simple experiments were performed to investigate a possible relationship between the ability to stimulate O_2 consumption and to increase the rate of MHb reduction. Such a relationship would be very helpful in assaying the slow MHb reductase enzymes because Heunnekins (12) found that the rate with O_2 as the final electron acceptor was about 7 times that when MHb was used.

II. Experimental

O_2 consumption was measured by the standard Warburg technique (25).

The reaction flask contained 2 ml of a volume to volume mixture of washed rabbit erythrocytes (see page 24, para. 2) and a saline incubation medium. The incubation medium contained: 6 meq / 1 of $MgCl_2$, 6 meq / 1 of K_2HPO_4 in a solution of 0.15 M NaCl, buffered with 0.01M Tris, pH 7.6. (The concentrations of Mg^{++} and phosphate were taken from the analysis figures in "Standard Values in Blood" (25)). The sidearm contained 0.2 ml of incubation medium fortified with 12.6 umoles of glucose and 1.3 umoles of one of the additive chemicals.

MHb reduction was determined as described under MHb reductase assays, Procedure I. Method B for MHb was used. Each ml of the reaction mixture contained 0.65 umoles of additive.

III. Results

The O_2 consumption of the untreated erythrocyte was essentially zero as reported by several authors (4,6,23). The addition of methylene blue and Vitamin K_3 produced a marked increase in the O_2 respiration. Ascorbic acid had little or no respiratory effect. See Table I.

Visual inspection of the reacting mixture showed varying effects. The erythrocytes treated with methylene blue showed no colour change at the end of the reaction period. This is to be expected as the dye colour would obscure any minor colour changes. Cells incubated with ascorbic acid were difficult to distinguish from normal cells for the first 2/3 of the reaction period, but then a green coloration due to the formation of choleglobin, began to appear (27). Cellular destruction was also evident. Vitamin K_3 treated cells steadily turned brown and eventually these cells lysed.

TABLE I

The Effect of Methylene Blue, Ascorbic Acid and Vitamin K_3 on the Oxygen Consumption of Rabbit Erythrocytes

Additive	Number of Determinations	Range of O_2 Consumption μl O_2/cells = 10 g Hb/100 ml
None	20	0 - 10
Methylene blue	20	125 - 175
Ascorbic acid	10	0 - 10
Vitamin K_3	20	120 - 140

Each determination contained 0.65 µmoles of additive per ml

TABLE II

The Effect of Methylene Blue, Ascorbic Acid and Vitakin K_3 on Methemoglobin Reduction in Rabbit Erythrocytes

Additive	Number of Determinations	1/2 Time of Reduction in min.
None	20	100
Ascorbic acid	5	30 - 50*
Methylene blue	5	7 - 10
Vitamin K_3	5	-**

Each determination contained 0.65 µmoles of additive per ml

* The range in the ascorbic acid figures is due to the formation of choleglobin which interferes with the MHb method.

** A reduction half-time cannot be given because Vitamin K_3 oxidizes Hb.

The MHb reduction rate in untreated MHb containing cells was first order, as reported by Jaffe (17). Methylene blue ascorbic acid increased the reduction rate but did not change the kinetics. A recent publication by Broberger, Ernster, and Zetterstrom (28) discusses this phenomenon. The MHb reduction data are compiled as Table II.

IV. Discussion

The results obtained from the study of O_2 exchange with Vitamin K_3 and methylene blue, and of MHb reduction with ascorbic acid and methylene blue agree favourably with those that would be expected from the data in the literature. Although methylene blue stimulates both respiration and MHb reduction, Vitamin K_3 stimulates only the respiration; and ascorbic acid, only the MHb reduction of erythrocytes. Therefore, a general correlation, between the effect of a drug on O_2 exchange and MHb reduction, cannot be made. Because there cannot be such a correlation, use of the stimulation of O_2 exchange by methylene blue for the study of MHb reductase is invalid.

THE METHEMOGLOBIN REDUCTASES OF THE ERYTHROCYTE

I. Introduction

Previous workers obtained no precise data concerning the nature of the MHb reductase sequences from studies of whole cell metabolism. Therefore, it was decided to start with the systems reduced one step to the hemolysate. Several reports of active hemolysate preparations have appeared (18,21,23). Substrates used by these workers include glucose-6-P, triose-P and a crude preparation of DPNH. In all cases reported, the reductase activity was lower than that found in the whole cell.

Two methods for following the MHb reductase reaction were developed. The first used the metabolic substrates, glucose-6-P, and triose-P, but when the optimal conditions for the reductase activities had been determined, it became apparent that this method was not sufficiently sensitive for our purposes.

An assay method that is to be used for inhibitor studies or during the isolation of an enzyme, must depend on the presence of only one enzyme in the test solution. If other enzymes are necessary, the inhibitor studies will be questionable as the enzyme which is being inhibited will be unknown. An isolation procedure may separate the enzymes required by an assay so that none of the fractions obtained will show activity. Therefore, to obtain information concerning the nature of the prosthetic groups of the MHb reductases by inhibitor studies and so that the method could be used during the isolation of the MHb reductases, enzymes other than MHb reductase had to be eliminated from the assay method. To achieve this, DPNH and TPNH were used as the substrates in the second method. Sampling

errors and the error in determining MHb were eliminated by measuring the reduction manometrically. MHb reductase D responded very well to the second method and some positive data were obtained. The method proved inadequate for studies of MHb reductase T but it was still possible to obtain some qualitative rate data.

The recent work of Perutz and co-workers (29) on the secondary and tertiary structures of Hb has some interesting implications towards MHb reductase. His structures indicate that the closest distance between any two heme groups is 25 A, and the average is nearer 30 A. If there is to be a simultaneous transfer of two electrons from the enzyme to the Hb molecule, the active site must enclose the distance between the hemes, and as the Hb molecule has an irregular surface with the hemes somewhat buried in folds, the normal lock and key theory of enzyme action (30) becomes a little strained when asked to cover this situation. There are other mechanistic possibilities, including the one electron transfer, and the simultaneous binding of two Hb molecules to the enzyme in the transition state. It was hoped that some simple kinetic experiments would show whether one or two molecules of MHb were involved in the activated enzyme complex. Although the data suggests that only one MHb molecule is involved, the evidence is far from conclusive.

Inhibitor studies were limited by the finding that the reductase D was sensitive to some unknown ion in the enzyme preparations, and by the instability of the reductase in solution. However, the studies indicate that sulfhydryl groups are involved in the reductase reaction.

II. General Methods

A. Total Hb and Total Protein

Total heme pigment was determined by the cyanomethemoglobin method of Drabkin (31). In this estimation, all heme pigment is converted to cyanomethemoglobin by the action of ferricyanide. The resulting colour was measured in the Klett-Summerson filter photometer with a number 54 Klett filter installed. The reagents used in the test are marketed by the Ortho Pharmaceutical Co. under the trade names of Acuglobin and Acudil.

The protein solutions used in this study were derived from erythrocytes in which about 97 per cent (32) of the protein is Hb. As the remainder of the cell contents are present, i.e. amino acids, coenzymes, etc., determination of protein by total N would have required long procedures if accuracy was wanted. As the normal optical methods for protein determination were inadequate because of the heme present, it was decided to estimate total protein as total heme pigment (33).

B. Methemoglobin in Hb - MHb mixtures

The MHb content of samples was determined by optical methods based on that described by Hunter (34). A short preliminary survey of the methods for MHb was made (35). The methods were judged for accuracy, speed, and ease of handling. Although the method of Evelyn and Malloy (36) appeared more accurate, the Hunter technique was chosen because it required no exacting dilutions and no additions to the coloured solutions. The assay tubes could be rapidly prepared as they did not require an exact amount of solution. In addition, the method was easily adaptable to the use of a nomogram for the calculation of the MHb concentration.

Two variations of the method were used.

Method A. This is the original method described by Hunter. The per cent MHb is measured in a solution of HbO_2 and MHb. Optical density readings are obtained in a spectrophotometer at 523 mμ (isosbestic)* and 630 mμ, a wavelength at which there is a large extinction difference between the two pigments. Samples were diluted so that the extinction at the isosbestic wavelength was approximately 0.6.

This, and any other method for MHb determination that does not employ some method for stabilizing the Hb, suffers from two drawbacks. The auto-oxidation of Hb by O_2 (37) is increased by the presence of salts (38) (curves A and B, fig. 3.). Thus, all dilutions must be made with demineralized water and buffers cannot be used. Therefore, the sample pH must be above 7.2 because the hemes of MHb act as an indicator (pK around 6.9). The absorption spectrum of the acid form does not contain the 630 mμ absorption band required for the optical methods.

Method B. In this modification of the method of Hunter (34), the Hb is stabilized by conversion to carboxyhemoglobin. The E_{520} (isosbestic, see fig. 4.) and E_{630} are determined. The pigment dilution is the same as in method A.

The sample source determined the procedure. Samples of erythrocyte suspensions were added to 2 ml of demineralized water to lake the cells. The tubes were gassed with CO, mixed, stoppered and set aside

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* An isosbestic wavelength is one at which two pigments have identical extinction coefficients. At these wavelengths the total pigment is determined and when the values obtained are compared to the optical density at a wavelength at which a large extinction difference occurs, the relative concentrations of the two pigments may be calculated.

for 5 min. Following this period, 5 ml of 0.05 M buffer, pH 7.4, was added from an automatic burrette. CO was used to raise the buffer from the reservoir of the burrette so that the buffer was saturated with CO at all times. After the buffer addition, the solutions received a second gassing, and were stoppered and centrifuged to remove any suspended material. The supernatant was decanted into 3 ml cuvettes and the E_{520} and E_{630}* read in a spectrophotometer using the Beckman micro attachment that was fitted. The instrument is described in the Appendix. II.

Hemolysate samples were added directly to the CO equilibrated buffer. Further treatment was identical to that given the buffered blood samples.

A nomogram was used for the calculation of the MHb content, from the photometer readings, as described by Hunter (34).

A modified method B lacked the disadvantages of method A. The addition of CO stabilized the Hb to oxidation for at least an hour, as shown in fig. 3, (curve C). Because the Hb had been stabilized, buffers could be used and the pH of the sample was no longer critical. The error in method A was $\pm$1 per cent in the range of 10 - 95 per cent MHb, when the method was used for determinations. In serial work, such as encountered in the MHb reductase assay I, the error quickly rose to $\pm$ 2.5 per cent. Method B proved reproducible to $\pm$0.5 per cent on single determinations, and in serial work $\pm$1 per cent could be obtained.

C. Carbon Monoxide Generation and Delivery

CO was generated by the reaction of sodium formate with

* Extinction or optical density at the subscript wavelength (mμ).

Fig. 3

Oxidation of Hb and HbCO by O_2

A Oxidation in 0.01 M NaCl
B Oxidation in demineralised H_2O
C Oxidation of HbCO

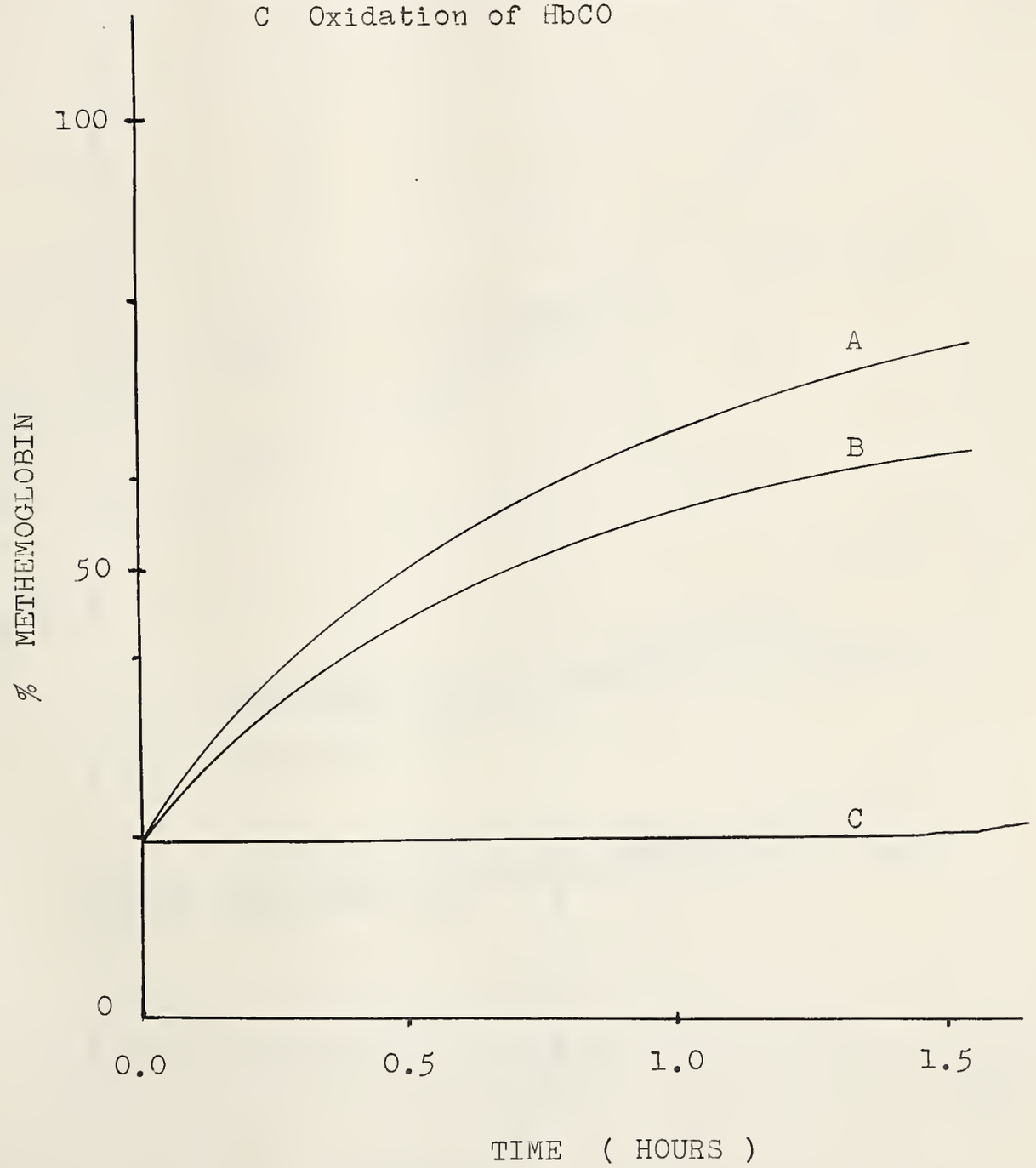


Fig. 4

Determination of Isosbestic Wavelength
Spectral Curves of HbCO and MHb

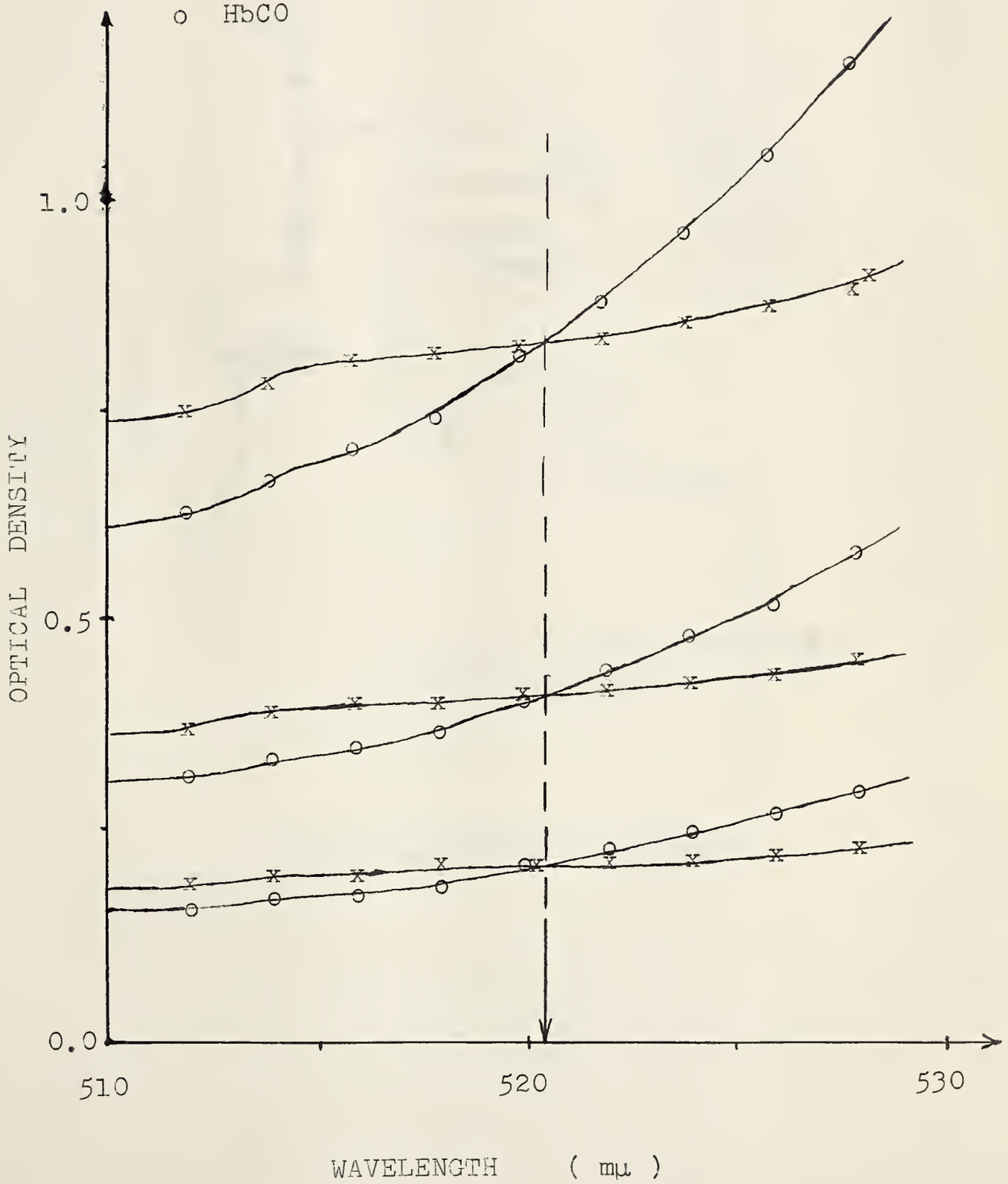
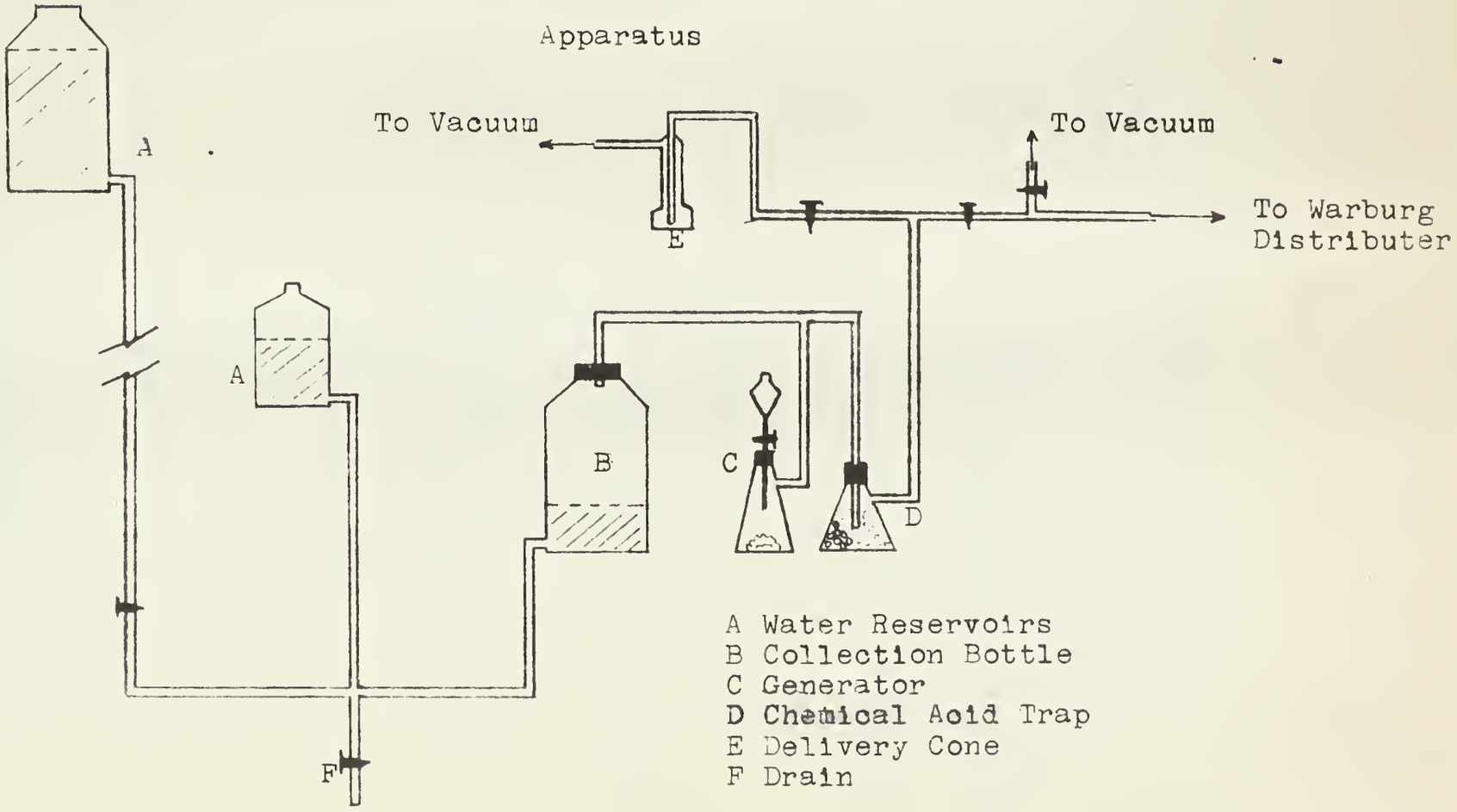


Fig. 5a

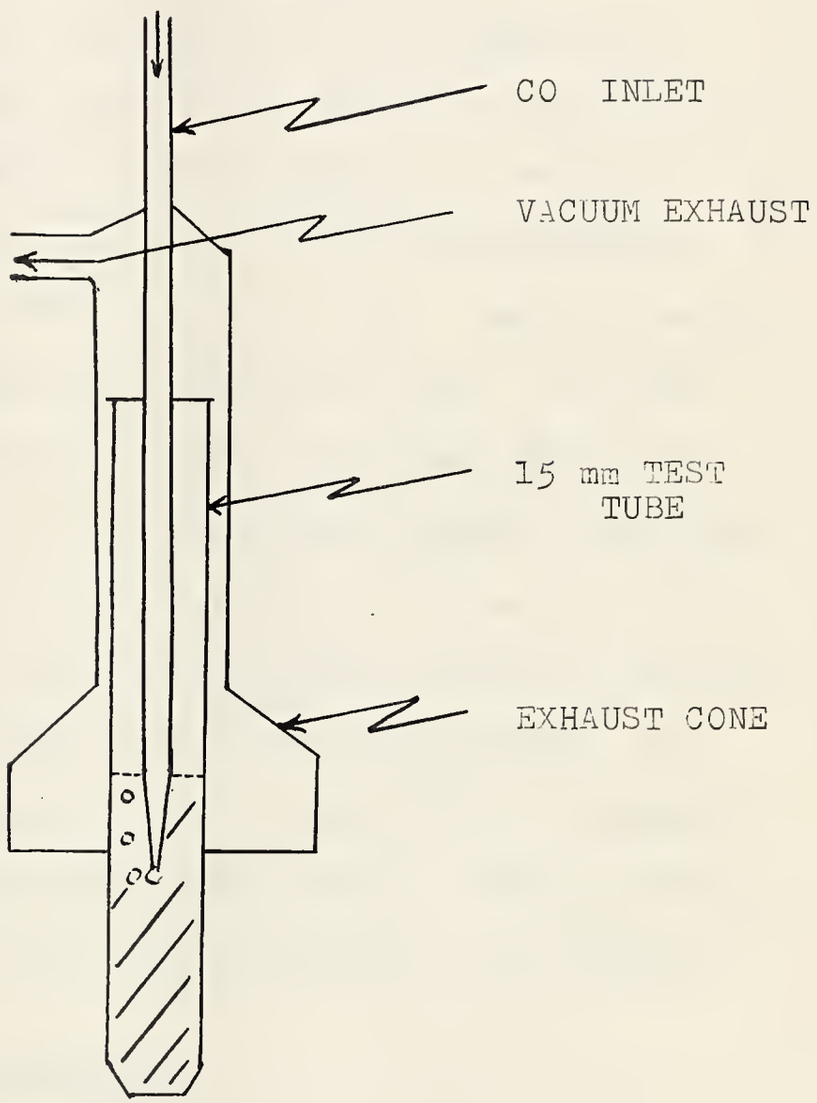
CO Generation and Delivery

Apparatus



A pressure head of 1 foot was provided by the lower reservoir and a head of 4 feet by the upper one. Without the trap, some difficulty was encountered with Formic acid volatilizing in the generation bottle and being delivered with the CO.

Fig. 5b



CO Delivery Cone

sulphuric acid. The gas was collected in an aspirator bottle by the downward displacement of water. Delivery of the gas to MHb determinations was made via the apparatus shown in fig. 5b. Fig. 5a shows diagramatically, the generation and delivery apparatus used.

D. Determination of Isosbestic Wavelengths

Solutions of equal concentration of heme pigments were prepared as follows. Normal whole cells were laked by the addition of 5 volumes of demineralized water. The stromatta were centrifuged out. Three 25 ml volumetric flasks were half filled with CO-equilibrated buffer, pH 7.4 and 1 mg of $NaNO_2$ was added to each. One half, one and two ml aliquots of the hemolysate solution were added. The flasks were gassed with CO and brought to volume with buffer. The optical densities of the solutions were determined over a range of wavelengths, using the narrowest possible band widths (0.01 - 0.03 mm slit with the Beckman micro attachment installed). Three more volumetric flasks were similarly prepared, with $Na_2S_2O_4$ used in place of the $NaNO_2$. The resulting graphs are presented in fig. 4. The isosbestic wavelength of the MHb, carboxyhemoglobin mixture was 520.5 mμ. (No correction for the wavelength error of the photometer was applied to this figure.).

III. Enzyme Preparations and Assays

A. Enzyme Preparations

1) Rabit blood, obtained by heart puncture, was collected in heparinized, isotonic saline. The suspension was centrifuged, and the supernatant removed by aspiration. The cells were then washed three times at the centrifuge with isotonic saline. The supernatant and buffy layer were removed each time by aspiration. The cells were suspended in

an equal volume of 0.15 M NaCl, buffered with 0.01 M Tris, pH 7.4, and used as normal whole cells.

2) Normal whole cells were incubated for 2 hours at 37°C with two volumes of isotonic saline, containing 0.342 g $NaNO_2$ per liter. After the incubation, the erythrocytes were washed at the centrifuge 5 times. The supernatant was removed by aspiration as before. The resulting cell preparation contained 60 - 70 per cent MHb, and is referred to as methemoglobinemic cells.

3) Hemolysate was prepared from normal whole cells by repeated freezing and thawing, followed by centrifugation in the cold to remove the stromatta and any suspended material. Hemolysate did not have a consistent MHb reductase activity although all preparations did show activity. The method used to hemolyse the cells seemed to have no bearing on the loss of MHb reductase activity. Hemolysates made by freeze thawing, by the action of saponin, and by osmotic hemolysis were equally affected. The hemolysate was also used in the study of erythrocyte dye reductase. Hemolysates made by the freeze thaw method and by the action of saponin had equal dyed reductase activity. The activity of osmotically hemolysed preparations was invariably lower. If the enzyme preparations required dilution or dissolving, the solution added had to be isotonic saline or a solution of at least equal tonicity, otherwise the protein precipitated and the enzymes were inactivated. The cause of this effect is unknown.

4) Lyophilizate was prepared from methemoglobinemic cells by freeze drying a one to one suspension of packed methemoglobinemic cells and unbuffered isotonic saline. The dried protein was stored at -20°C until required.

B. Methemoglobin Reductase Assays

Procedure I in which a reaction mixture was prepared in open tubes and samples were taken for MHb assay at various time intervals.

Reaction tubes contained 1 ml of lyophilizate, 1 ml of the appropriate buffer solution, and 1 ml of the proper substrate solution. The enzyme and buffer were mixed and preincubated simultaneously with the substrate solution at 37°C. When the reaction was started by the addition of substrate, a sample was taken for determination of the initial MHb concentration. Samples of appropriate size (usually 0.2 ml) were taken at 10 min. intervals for 40 min. and a final sample after 60 min. Total pigment was determined following the reaction period. The per cent MHb in each sample was estimated and the resulting figures plotted against time. When necessary, the moles of MHb reduced per unit time were calculated from the per cent reduction and the total pigment concentration.

Two substrate solutions were used. Each MHb reductase D assay contained: GSH - 10 μmoles, DPN^+ - 4.5 μmoles, ADP - 1.4 μmoles, glyceraldehyde-3-P - 20 μmoles, Mg^{++} - 3 μmoles. MHb reductase T assays contained: TPN^+ - 4.8 μmoles, glucose-6-P - 15 μmoles. The buffers employed were 0.05 M Tris in the MHb reductase T assay and 0.05 M phosphate in the DPN^+ coupled reductase assay. The Tris buffer was used in the MHb reductase T assay because the oxidation of glucose-6-P to 6-P-glucuronic acid is inhibited by high concentrations of phosphate (39).

Several difficulties were presented by this assay procedure. The reaction produced a large amount of hydrogen ion, which necessitated the use of relatively high buffer concentrations. The acid originates from all parts of the reaction sequence. Four electrons are required to

reduce one molecule of MHb. Each pair of electrons passed from substrate to MHb by the enzyme chain, is accompanied by the release of two hydrogen ions. Hence, for each mole of MHb reduced four moles of hydrogen ion appear. There is an unknown amount of acid produced by the further metabolism of the substrate, after the pyridine nucleotide has been reduced. There is a shift in the isoelectric point of the Hb molecule coincident with its reduction. As the protein forms one of the main buffer constituents of the reaction mixture, the reduction will also have an effect on the pH of the solution. During the hour reaction period, the pH of the mixture shifted one unit when 0.01 M buffer was used. With the 0.05 M buffer used in the assays, the pH drop was still 1/2 a unit.

The minimum interval for sampling was 7 min., due to the time involved in MHb determination. As a result, a maximum of 6 samples could be taken during the first, critical 40 min. of the reaction period. The most consistent results were obtained with a 10 min. sampling interval, but this produced only four points for the rate determination.

The error of method A for MHb was so great that a rate variance of $\pm$20 per cent was common. Method B lowered the rate error considerably but for the purposes for which the assay was intended, the error of $\pm$5 per cent was still too great. However, the assay was useful to demonstrate the presence of the reductases, and to give a qualitative index of the reduction rate.

Procedure II in which the reduction of MHb was followed by the uptake of CO or O_2 as it combined with the newly reduced Hb.

A typical reaction mixture consisted of 1 ml of a 10 g per cent solution of lyophilizate in reaction buffer, placed in the main chamber

of a Warburg flask. The reaction buffer was 0.05 M phosphate buffer that was 12 mg per cent in EDTA. The substrate solution was a 1:1 mixture of reaction buffer and a 20 mg per ml solution of DPNH in 0.01 M NaOH. To start the reaction 0.2 ml of the substrate solution was added from the flask sidearm. The center well contained 0.2 ml of 7 per cent KOH absorbed on a small piece of accordion folded filter paper. Two methods of gassing were used, neither of which affected the rate of MHb reductase D to any extent. Total gas exchange was accomplished by a series of 5 vacuum exchanges with CO. Cursory gassing was done by flowing a stream of CO into the manometer mounted Warburg flask for half a min. This was sufficient to replace the Hb bound to O_2 with CO and leave some CO in the gas phase.

The Warburg apparatus was temperature regulated at 25 $\pm$0.5°C. The stroke rate was 110 per min.

After a 20 min. equilibration period, the fluid level in the manometer was adjusted and the taps closed. The substrate was tipped in after another 20 min. period. Readings were taken at 10 min. intervals for 90 min. and then, at longer intervals up to 180 min. After the reaction period, the reaction mixture was sampled for determination of total Hb.

This assay was the best for MHb reductase D that has been devised to date. Warburg (4), Gibson (8) and others all used some variation of this technique to follow MHb reductase. All other assay methods are affected by the auto-oxidation of diluted heme samples by O_2. The stoichiometry is most favourable for the slow reductase reaction as the ratio of moles taken up to moles MHb reduced is 4, according to the reaction:

$$MHb + 4\,CO + 4\,e^- \rightleftharpoons Hb(CO)_4$$

As the reaction proceeds under an inert gas when total gas exchange is used, the oxidation of Hb by O_2 does not occur, and the rate obtained is a true rate. Because there are fewer reactions involved when reduced pyridine nucleotides are used as the substrates, the extensive pH shift encountered with Procedure I does not occur with this method.

Procedure II proved unsatisfactory for MHb reductase T assay as this enzyme appears sensitive to CO. (see Table X). However, with cursory gassing, the presence of the T-enzyme may be demonstrated and, using air as the gas phase, some indication of the rate of this activity may be obtained. The rate obtained with O_2 present is the result of the reduction rate and the auto-oxidation rate. In the following experiments total gas exchange was used except where otherwise stated.

IV. Results

A. Procedure I

1) Optimal Concentration of Reactants

The optimal concentrations of reactants were determined for both the MHb reductase D and T assays. The preliminary experiments were performed using the concentrations of cofactors and ions as reported in "Standard Values in Blood" (25). When the optimal requirements were found, the work was repeated, varying the reactant concentrations one at a time. About half way through this work, new DPN^+ and ADP were obtained and the reaction was stimulated by these new chemicals. For this effect, compare the highest rate in Tables IV and IX. The reason for the stimulation is unknown. It is unlikely that the first lot of chemicals had deteriorated, as the optimal concentrations of the cofactors were the same with both lots of material. In view of the

later finding that the MHb reductase D is inhibited by some ion, it is a possibility that the first batch of ADP and/or DPN^+ contained some of the inhibitor ion, but as there was none of the first batch of the chemicals left, this idea could not be confirmed.

No effect of adding Mg^{++} could be demonstrated but the results were more consistent when 3 µmoles of Mg^{++} were added to each reaction mixture.

TABLE III

Effect of DPN^+ Concentration on the

Velocity of MHb Reductase D

DPN^+ Concentration (final M x 10^3)	Per Cent MHb Reduced per hour
0.38	7
0.75	10
1.13	15
1.50	20
2.25	20

TABLE IV

GSH Concentration and the Velocity

of MHb Reductase D

GSH Concentration (final M x 10^3)	Per Cent MHb Reduced per hour
0.33	-
1.11	5
2.22	10
3.33	20
6.67	20

TABLE V

Effect of Glyceraldehyde-3-P Concentration on MHb Reductase D

Glyceraldehyde-3-P Conc. (final M x 10^3)	% MHb Reduced per hour
1.67	20
3.33	25
6.67	30
10.0	30

TABLE VI

Effect of ADP Concentration on

MHb Reductase D Activity

ADP Concentration (final M x 10^2)	% MHb Reduced per hour
0.367	24
0.40	33
0.467	35
0.92	35

The optimal concentration of GSH was 3.33×10^{-3}M, or about 100 mg per cent. The values reported in the literature for the GSH level in erythrocytes are around 50 mg per cent (40), except for the recent findings of Collier and Tsen (41) who reported GSH levels around 100 mg per cent. One would expect the erythrocyte level of GSH to be optimal for the enzymes within the cell that required the substance. Except for the one study mentioned, in which a different analytical procedure was used, the values reported in the literature are only half the optimum.

TABLE VII

Effect of Glucose-6-P on the

Rate of MHb Reductase T

Glucose-6-P Concentration (final M x 10^3)	Per Cent MHb Reduced per hour
1.67	9
3.33	17
5.0	23
6.67	23

TABLE VIII

TPN^+ Concentration and the Rate of

MHb Reductase T

TPN^+ Concentration (final M x 10^3)	Per Cent MHb Reduced per hour
0.43	15
0.84	21
1.67	25
2.3	24

The true final concentration of all materials will be higher than the quoted figures because of material present in the lyophilizate used as the enzyme source. Similarly, the reaction rate will not be zero when one of the components is omitted.

TABLE IX

Optimal Concentration of Cofactors and Substrates

Involved in MHb Reductase Assays: Procedure I

MHb Reductase	Substrate or Cofactor	Optimum Conc. (final M x 10^3)	M Concentration Used x 10^3
D	DPN^+	1.50	1.50
	GSH	3.33	3.33
	GlAl-3-P*	6.67	20.0
	ADP	0.50	0.50
T	TPN^+	1.6	1.60
	Glucose-6-P	5.0	15.0

*GlAl-3-P = Glyceraldehyde-3-P

2) The Reaction Rates

The rates of MHb reductase D and T were of the same order of magnitude. Fig. 6 shows typical MHb reductase rate curves. The solid line in each case is the best zero order rate curve, and the broken line the best first order curve for the data. No distinction can be made between the two rate orders from the data available. For convenience, the rates were taken as zero order. The error introduced in the rate determinations by the error inherent in the MHb determinations was large (20 - 30%) as explained in the description of the assay. Data in fig. 6a were obtained using the MHb method A. At the 90 min. mark, the difference between the two extreme points is 18 per cent MHb. The mean and standard deviation of the points are $39.9 \pm 5.0\%$ which represents a rate variance of 25 per cent. For the data in fig. 6b, MHb determination B was used. Here the rate variance, at time 60, is 9 per cent.

The average reduction rate for 30 determinations were:

MHb reductase D.....0.05 μmoles MHb / 10 min.

MHb reductase T.....0.043 μmoles MHb / 10 min.

These figures were calculated from the average rate of MHb reduced per hour (30% for MHb reductase D and 25% for the T enzyme) for a protein concentration of 10 g per cent and an initial MHb level of 70 per cent. For the purposes of the calculation the molecular weight of Hb was taken as 67,000.

3) Rate Experiments using DPNH and TPNH as Substrates.

For these experiments, the same procedure was followed as before except that the substrate solution was a 1 mg / ml solution of

Fig. 6a

MHb Reductase D Activity
Rate Graph: Procedure I

Graph of % MHb vs time

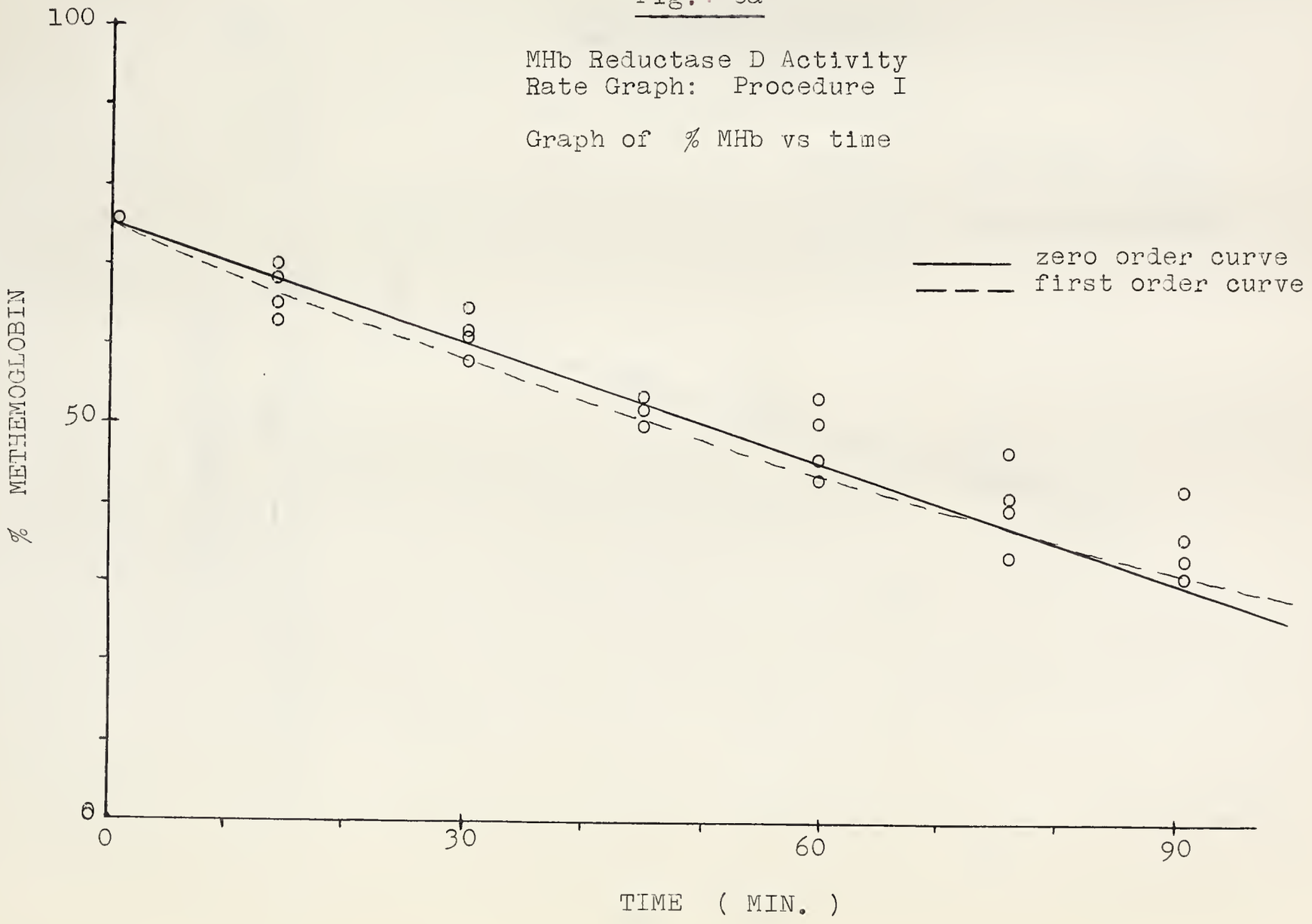


Fig. 6b

MHb Reductase T Activity
Rate Graph: Procedure I

Graph of %MHb vs time

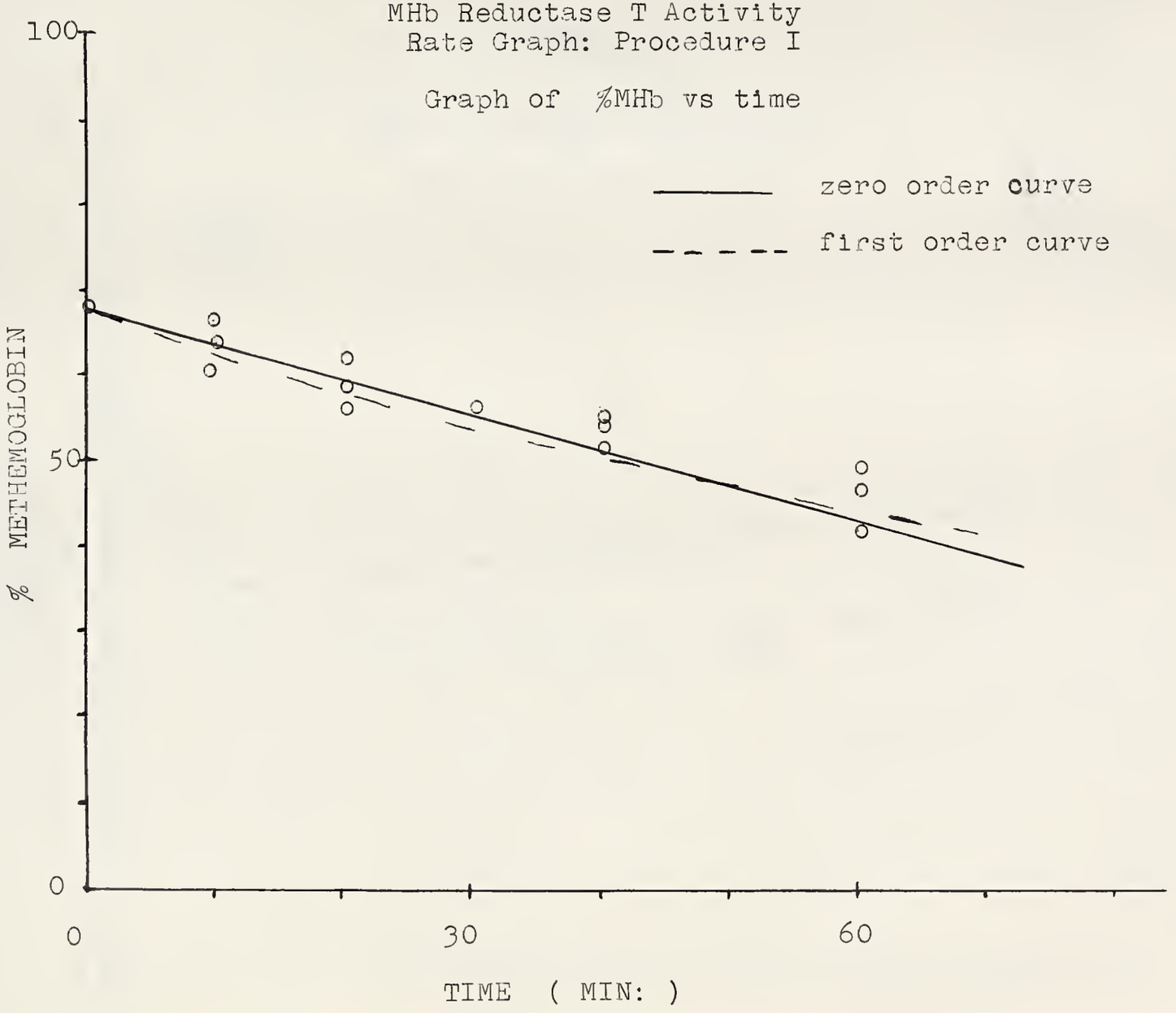
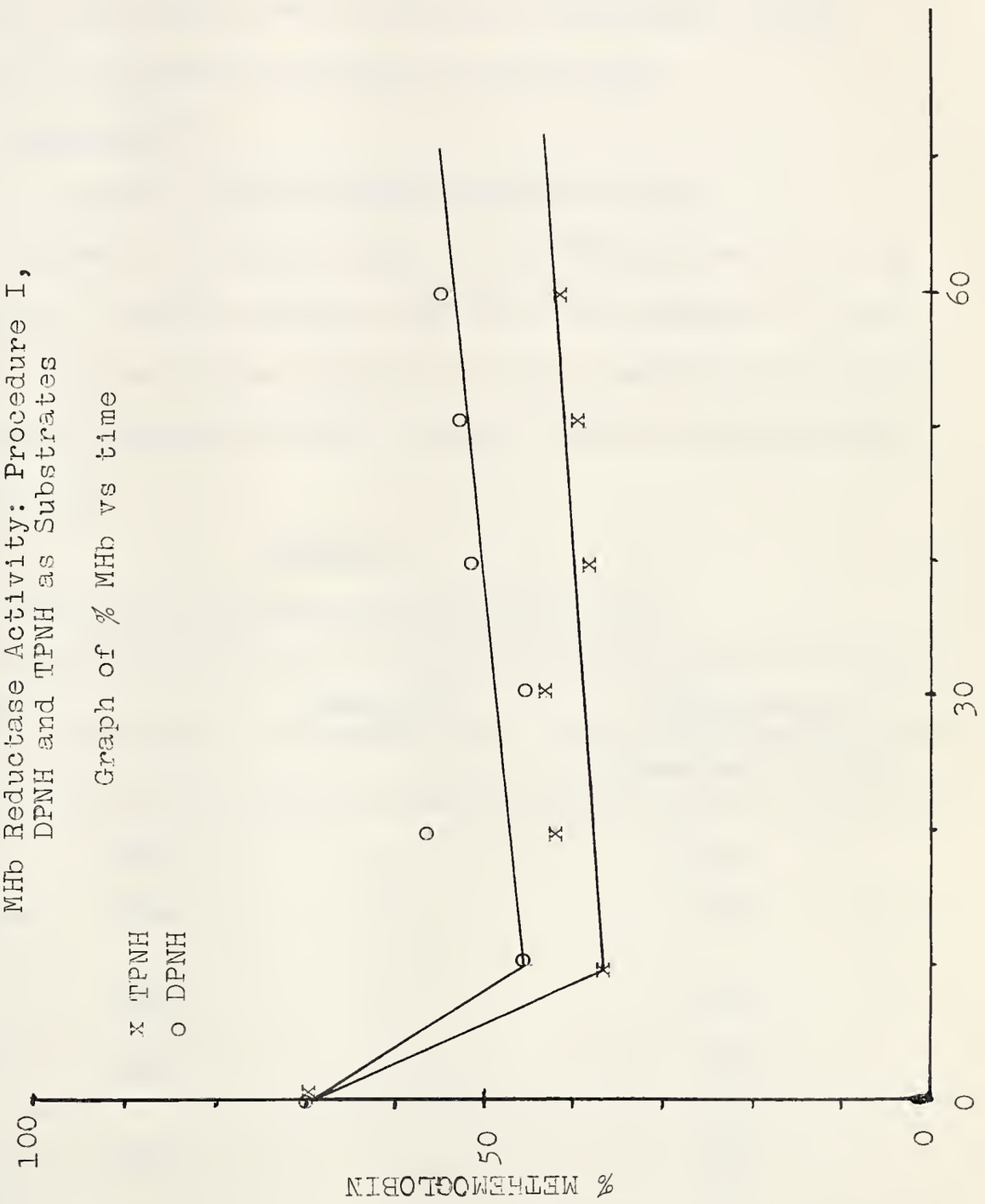


Fig. 7

MHb Reductase Activity: Procedure I,
DPNH and TPNH as Substrates

Graph of % MHb vs time



reduced pyridine nucleotide in reaction buffer. Fig. 7 shows the result of this experiment plotted on the same scales as used in fig. 6. The reaction was essentially complete in the first 10 min. Both rates were faster than those found in the previous experiments in which glucose-6-P and triose-P were used as the substrates.

B. Procedure II

1) Effect of CO on the MHb Reductases D and T

CO had no inhibitory effect on the MHb reductase D activity. MHb reductase T was 88% inhibited under CO at one atmosphere. Cursory gassing produced an average inhibition of 35%. Therefore, Procedure II was considered inadequate for MHb reductase T assay, even when O_2 was the exchange gas.

TABLE X

Inhibition of Methemoglobin Reductase by CO

CO gassing procedure	Substrate	Number of determinations	Reduction rate μmoles MHb/10 min/ml soln.* (numerical average)
No CO Air as gas phase	TPNH	5	0.170
	DPNH	4	0.088
Cursory	TPNH	4	0.060
	DPNH	3	0.090
Complete exchange	TPNH	4	0.019
	DPNH	4	0.091

Enzyme preparation I was used for this study

* The determinations were done on a 10 g per cent solution of lyophilizate.

TABLE XI

Rates of the Methemoglobin Reductases of Rabbit Erythrocyte;

Enzymes Acting Both Singly, and Together

Enzyme preparation	Substrate	Number of Estimations	Reduction Rate μmoles MHb/10 min/ml soln. ± Standard deviation	Relative Reduction Rate
I	DPNH	16	0.0909 ± 0.0053	1.00
	TPNH	2	—	1.44
II	DPNH	8	0.1310 ± 0.0206	1.44
	TPNH*	5	—	1.70
	BOTH*	2	—	2.75
	BOTH"	3	—	1.90

* Results with air as gas phase

" Reaction under cursory gassing

Relative figures are given because the TPNH assays could not be corrected for the auto-oxidation of Hb by O_2. As well, insufficient data were available to permit a statistical treatment of the rate figures.

If one compares the MHb reductase D activity when air was the gas phase, and when CO was the atmosphere, there is a difference of 0.004 μmoles MHb / 10 min. in the reduction rates. This is due to the action of O_2 upon Hb and constitutes a 4.4% error in the reduction rate. In the MHb reductase T assay the higher reduction rate would make this error smaller but because of this inherent error and because of the high cost of purified TPNH only the simplest rate studies were done on MHb reductase T.

2) Rates of Reduction

The rates quoted were determined on a 10 g per cent solution of lyophilizate. The figure of 10 g per cent was convenient because the viscosity of the solution was not prohibitive when Ostwald pippettes were used for the measuring of the solutions, and there was ample enzyme and substrate in this solution to produce easily measurable changes on the manometers.

Two batches of lyophilizate were prepared. The specific activities of the two were close, the second being slightly more active, albeit with a greater statistical variance. See Table XI.

Each assay contained 2 mg of DPNH or TPNH. When both substrates were present, 2 mg of each were added to the assays.

The figures for MHb reductase T are given as relative values, as are the rates when both substrates were present. The MHb reductase D activity of the first preparation was taken as unity and the other data expressed relative to it. Only relative figures are given because the T enzyme must be measured under an O_2 atmosphere and no correction could be made for the auto-oxidation of Hb by O_2. As well the range of values for the reductase reactions necessitated the use of statistics and there were

insufficient data for statistical calculations on the MHb reductase T rates. Hence, the rates found for MHb reductases D and T are not strictly comparable and to prevent comparisons that are not correct, the data were given as relative values.

The rate of MHb reductase T was faster than that found for MHb reductase D. When both DPNH and TPNH were added to the reaction mixture, the reduction rate was always greater than that found when either substrate was used singly under the same conditions.

3) Activation by EDTA

The lyophilizate which was used as a source of the enzyme, contained all the ingredients present in the whole cell. As there are a large number of metal ions present in blood, the effect of a sequestering agent was determined in case there were traces of an inhibitor ion present. A significant activation occurred when EDTA was added to the reaction mixture. EDTA concentrations of 20 and 30 mg per cent produced no further increase in the reduction rates.

TABLE XII

EDTA Activation of MHb Reductase D

Preparation	Concentration of Versene	Number of Determinations	Rate of Reduction µmoles MHb/10 min./ml soln.
I	None	5	0.050
	10 mg %	16	0.091
II	None	5	0.0163
	10 mg %	8	0.131

A 10 g per cent solution of protein (lyophilizate) was used in these experiments.

4) The Possibility of Transhydrogenase Activity

The possibility that the MHb reductase activity exhibited with one of the pyridine nucleotides is due to the presence of a transhydrogenase has been mentioned in the General Introduction. The lyophilizate that was used as the source of the enzyme and MHb contains the coenzymes required for transhydrogenase activity (TPN^+ and DPN^+), although not necessarily in the optimal concentrations. The reduction rate produced by a sequence that starts with a transhydrogenation can equal, but never surpass the rate found when the transhydrogenase has been eliminated from the sequence. For this reason, it is unlikely that the MHb reductase T activity is due to a TPNH to DPN^+ transhydrogenation. However, the MHb reductase D activity is lower and so could be the result of a sequence such as:

$$DPNH \xrightarrow{e^-} TPN^+ \xrightarrow{e^-} \text{Reductase T} \xrightarrow{e^-} MHb$$

If such were the case, it is unlikely that the TPN^+ concentration would be optimal because the system has been diluted from that found in the cell. One would expect that the addition of TPN^+ to the system would result in an increase in the MHb reduction rate found with DPNH.

Two series of experiments were performed to test for transhydrogenase activity. The two series differed only in the time at which the TPN^+ was added to the reaction. In series I, the TPN^+ was added to the enzyme solution and in series II (carried out in double sidearm flasks), the TPN^+ was added from the second sidearm, 30 min. after the addition of the substrate. In all cases, 2 mg of TPN^+ were added to each assay. The gas phase was air, to prevent any interactions with CO.

TABLE XIII

The Effect of TPN^+ on the Rate of MHb Reductase D:
A Test for Transhydrogenase

Series	TPN^+ µmoles	Number of Determinations	Reduction Rate µmoles MHb/10 min./ml soln. (Numerical Averages)	
			normal	with TPN^+
I	1.25	6	0.091	0.110
II	1.25	6	0.117	0.118

The protein concentration in these experiments was 10g %.

The reaction rate in series I was slightly increased by the addition of TPN^+. Because the TPN^+ was in the reaction mixture during the equilibration period, it is possible that some TPNH was formed by the reaction of endogenous substrates, and the reaction rate in this case is the result of the normal DPNH reduction and a slow MHb reductase T activity.

The results obtained indicate that there are, indeed, two MHb reductases and that MHb reductase D activity is not the result of a transhydrogenation followed by reduction. There may be some transydrogenation occurring, but the amount is not sufficient to cause a serious error in the reductase assays.

5) MHb Reductase D; Substrate Concentrations

a) Effect of DPNH concentration on the Reductase Rate

In these experiments, the DPNH concentration was varied 20 fold. The reduction rate was independent of the DPNH concentration when it was above 5×10^{-4}M. The maximum amount of MHb that may be reduced by a given amount of substrate is one half the amount of DPNH present. The reaction

follows the equation:

$$2DPNH + MHb \rightleftharpoons 2DPN^{+} + Hb$$

When the rate begins to show an effect of lowered DPNH concentration i.e. DPNH less than 5×10^{-4}M, the total reaction rapidly drops below the limits of sensitivity of manometric methods. This limit is generally accepted to be a change of 10 µl in a maximum period of one hour. The limit is the equivalent of 0.112 µmoles of MHb reduced in the same period. It may be seen from Table XIV that the limit has been reached when the DPNH concentration is 2.1×10^{-4}M.

The DPNH concentration at which the rate becomes independent of the DPNH concentration is only twice this figure. Therefore, a K_m determined by our method would be meaningless.

The substrate concentration used in the assays was 10.7×10^{-4}M. With this concentration, sufficient manometer readings could be obtained to produce accurate rate graphs. Also, this gave a steady reaction rate for a period of time that permitted an estimation of the effect of materials added after the reaction had been proceeding for some time.

TABLE XIV

DPNH Concentration and the Rate of

MHb Reductase D.

DPNH (final M x 10^4)	DPNH µmoles present /assay	Reduction Rate (µmoles MHb/10 min. /ml of soln.)	Total µmoles of MHb reduced
42.6	5.11	0.0625	2.00
21.3	2.56	0.064	1.26
10.7	1.28	0.059	0.64
5.33	0.64	0.063	0.32
2.13	0.25	0.03	0.12
1.00	0.12	- unmeasurable	-

The protein concentration in these assays was 10 g %.

b) The Effect of Protein Concentration on the Reduction Rate.

MHb reductase D has two substrates: DPNH and MHb. It has been shown that the specific rate constant for the reduction is independent of the DPNH concentration under the assay conditions. The rate is dependent upon the MHb concentration but to determine the nature of this dependence is not a simple task. There are several mechanisms that the reduction may follow that require different amounts of MHb in the active complex of the enzyme. Experimentally these mechanisms are almost indistinguishable.

The active complex may include one MHb molecule, or two, and the rate expression may not show the effect of the binding of the two MHb molecules. The mechanism may be such that one MHb is rapidly bound and the second, much more slowly. In this case, the binding of the first

molecule can only be studied by transient state kinetics. With the experimental methods used, this type of mechanism cannot be distinguished from that in which only one MHb molecule is bound. However, if the mentioned mechanism is correct, the effect of the binding of the first molecule of MHb should be noted as a slight shift of the kinetics to a higher order when the reaction mixture is diluted.

In the procedure, the enzyme and substrate MHb are present in the protein added to the assay, and so presumably, they will be in a constant ratio in the same batch of lyophilizate. Thus, if the system is diluted, the effect of the dilution of the enzyme and substrate MHb will be a function of the concentration of the protein present in the assay.

The protein concentration was varied from 0.35 g per cent to 130 g per cent in two groups of assays. The results were plotted in several different ways. Table XV contains the experimental data and the calculated figures for the graphs in fig. 8, 9 and 10. The data gave a linear curve when plotted as the activity vs. the square of the g protein present (fig. 9). A statistical regression line was calculated (42) and the resulting equation was:

$$V = 4.60\ (\text{g protein})^2 + 0.0039 \quad 2/V = \text{velocity}$$

As a check, the graph in fig. 10 was prepared and a regression line calculated. The equation was:

$$\frac{V}{\text{g protein}} = 4.35\ (\text{g protein}) + 0.0656$$

Theoretically, the two should differ only in the values plotted. The slopes should be the same and the intercept, (0,0). That these conditions are not fulfilled is an indication that there are deviations from the theoretical line. The third graph, i.e. fig. 10, tends to magnify such deviations at the low protein concentrations. There are several

TABLE XV

Effect of Protein Concentration on Methemoglobin Reductase D

Reduction Rate µmoles MHb/10 min/ml soln.	g Protein present in assay	g Protein squared (x 10^3)	Reduction Rate / g protein
0.1670	0.1299	27.9	0.778
0.1675	0.1354	28.05	0.808
0.1450	0.1019	20.7	0.703
0.1220	0.0719	14.9	0.589
0.1150	0.0594	13.2	0.517
0.0924	0.0444	8.55	0.481
0.0750	0.0294	5.38	0.392
0.0515	0.0169	2.62	0.328
.....*			
0.0955	0.0450	9.12	0.471
0.0525	0.0150	2.76	0.286
0.0452	0.0120	2.04	0.265
0.0325	0.0060	1.06	0.185
0.0236	0.0035	0.56	0.148

*The results above this line were used to calculate the regression line in fig. 9,10. Results below the line are from a different experiment in which 2 x volume of reaction mixture was used to give more accurate results at lower levels of gas exchange.

Fig. 8

Effect of Protein Concentration on
the Rate of MHb Reductase D

Plot of g protein vs activity

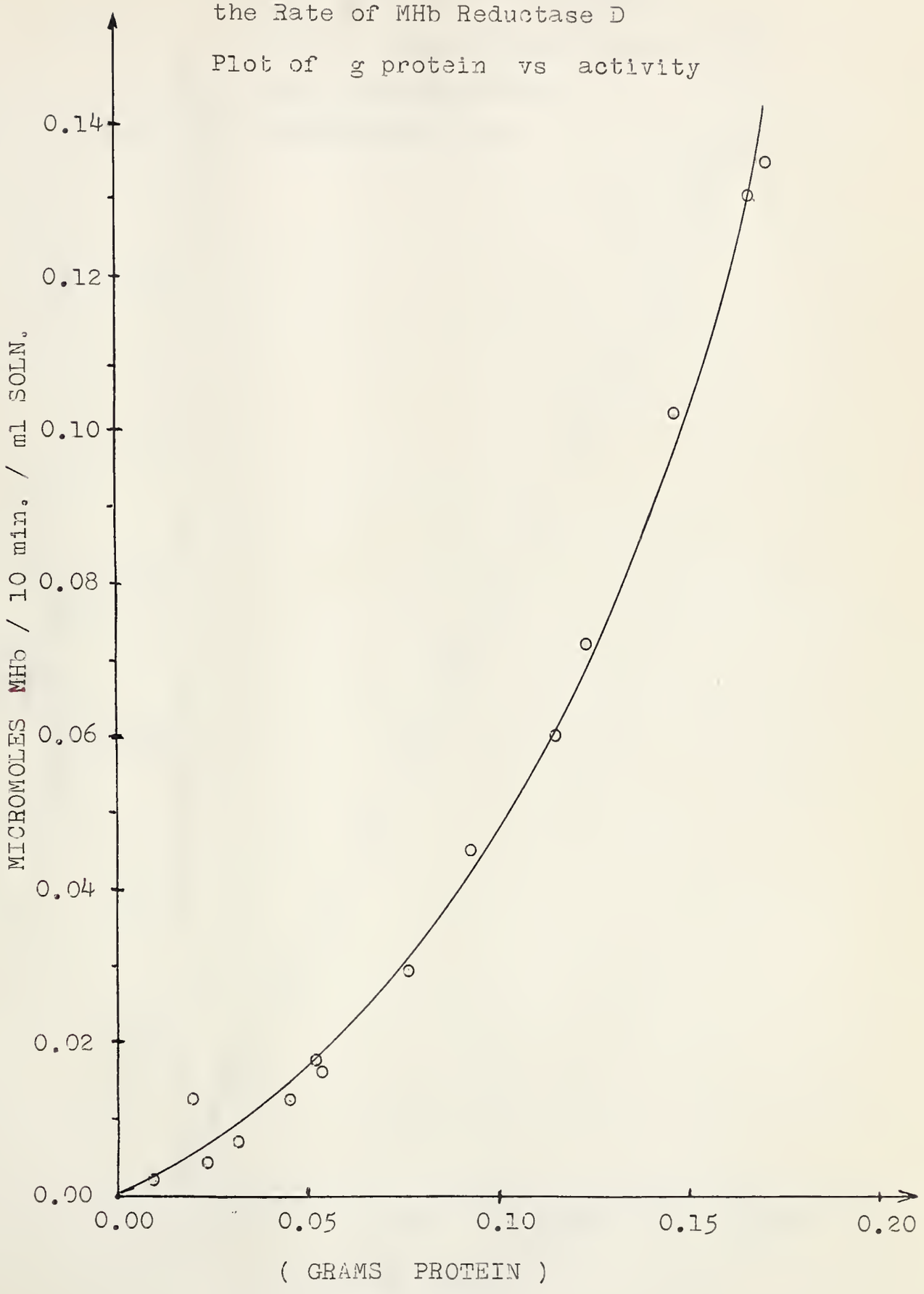


Fig. 9

Effect of Protein Concentration on the Rate of MHb Reductase D

Plot of activity vs $(g\text{-protein})^2$

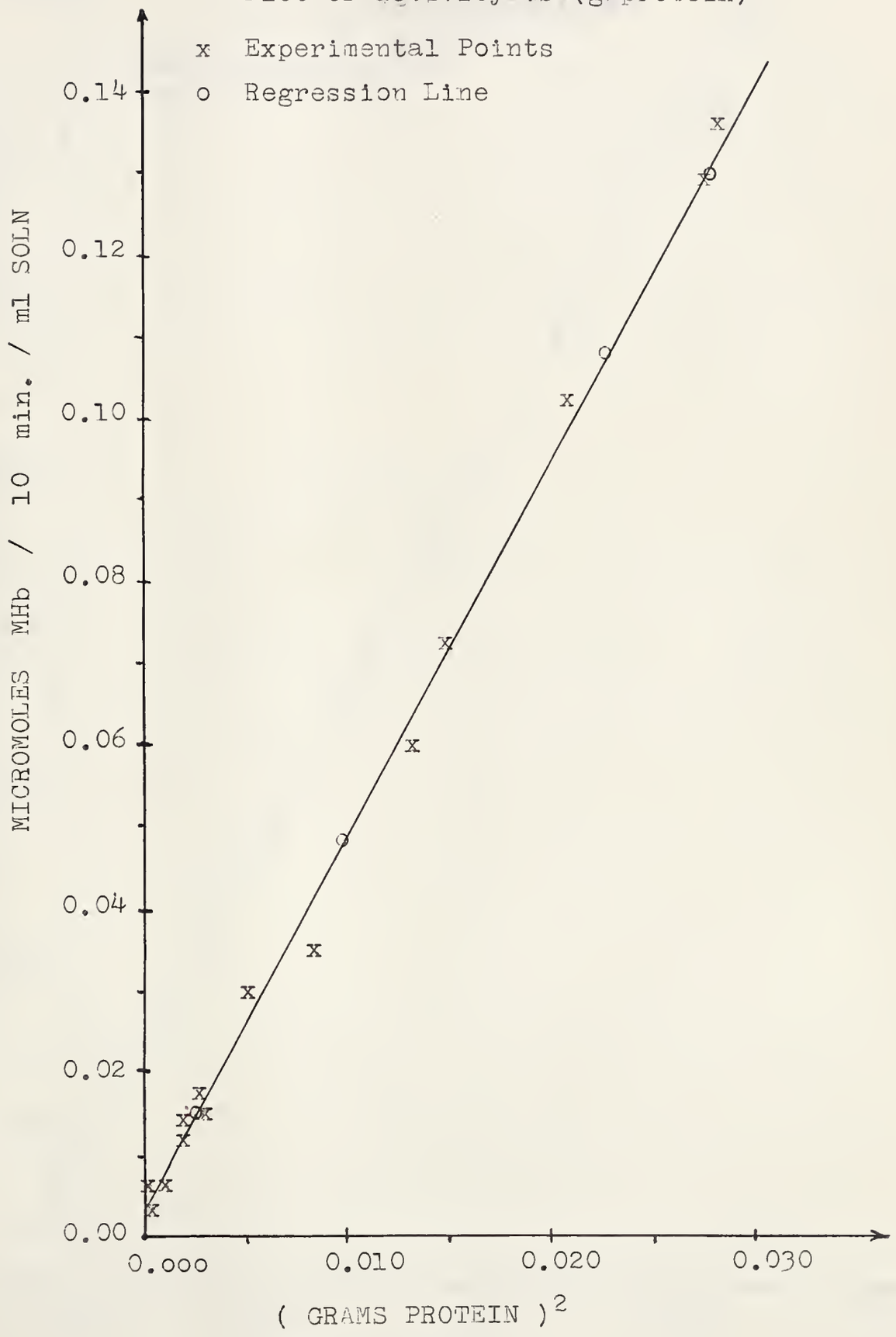
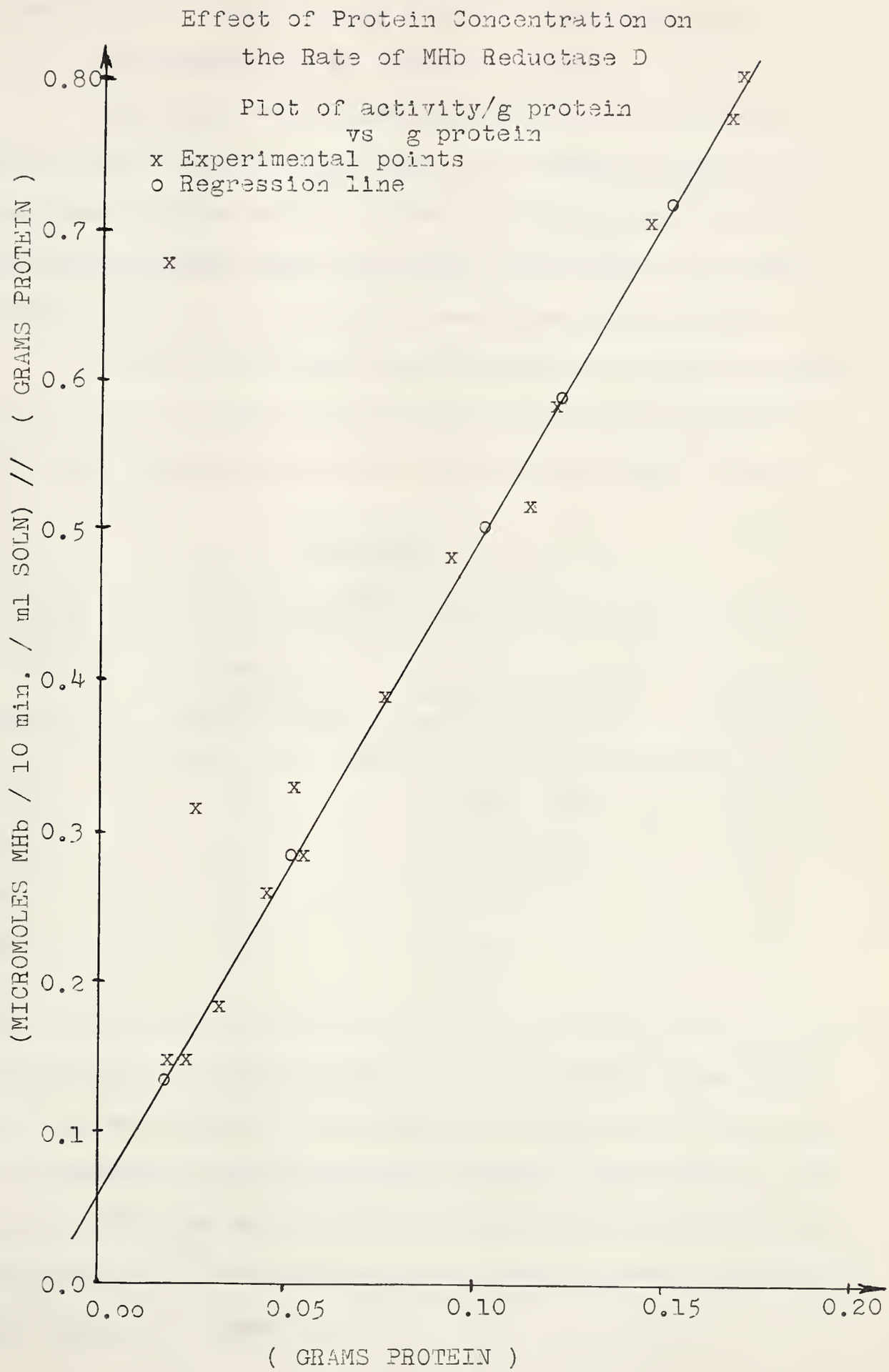


Fig. 10


Effect of Protein Concentration on
the Rate of MHb Reductase D
Plot of activity/g protein
vs g protein
x Experimental points
o Regression line
(MICROMOLES MHb / 10 min. / ml SOLN) // (GRAMS PROTEIN)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.80
0.00
0.05
0.10
0.15
0.20
(GRAMS PROTEIN)

explanations for these deviations that will be discussed later.

6) MHb Reductase D; Inhibitor Studies

Erythrocyte MHb reductase D is sensitive to a variety of materials, which diminish its activity. Anti-malarial drugs such as quinine and Atabrine, and the sulfhydril coupling agent, p-chloromercuribenzoate(PCMB) inhibit the enzyme. With PCMB, the SH groups of the Hb present have to be considered when interpreting the data.

At the pH of the assay, heavy metals precipitated the proteins so the effect of the Hg^{++} and other heavy metal poisons could not be determined. Iodoacetamide and N-ethylmaleimide were also tried as

TABLE XVI

Inhibition of MHb Reductase D

Inhibitor	Concentration of Inhibitor (final M x 10^4)	Rate of Reduction µmoles MHb/10 min.	Per Cent Inhibition
Quinine	4.0	0.025, 0.023	72, 75
Atabrine	4.0	0.026, 0.023	71, 75
PCMB	16.5	0.050	45
PCMB	5.0	0.090	0
None	-	0.091	-

inhibitors, but the time required for the reaction with Iodoacetamide caused the inactivation of the enzyme and precipitation of the Hb, and the concentration of N-ethylmaleimide required to cause inhibition was so high (10^{-2}M) that the inhibition was questionable on grounds of concentration alone. The N-ethylmaleimide is not as reactive towards sulfhydryl groups as the PCMB.

V. Discussion

There has been disagreement among research workers as to the pyridine nucleotide involved in MHb reduction. Heunnekins and co-workers (12) think that TPNH is utilized while Gibson (8) believes that DPNH is the coenzyme of reduction. Both groups are correct as there are two MHb reductases present each requiring a different pyridine nucleotide. Kiese's (13) suggestion that two reductases are present has not previously been confirmed. Bodansky and co-workers (18) agree with Kiese and the point was again presented in a review (1) but again the work was not satisfactorily confirmed.

The question arose as to whether there was one enzyme using both DPNH and TPNH or whether there were two enzymes, each specific for one of the pyridine nucleotides. The reduction rates for both MHb reductase D and T are reported in Table XI. In this table there are included the results of experiments in which both DPNH and TPNH were present in the same reaction mixture. Had there been only one enzyme, using both pyridine nucleotides as substrates, this latter rate would have been equal to the more rapid reaction with a single substrate present. The velocity with both substrates present was greater than the rate with either single substrate, and, when air was used as the gas phase, the velocity was almost the sum of the rates when single substrates were employed. This relationship can only be explained if two MHb reductases are present in the erythrocyte.

The sensitivity of only one of the MHb reductases to CO adds further confirmation to the hypothesis that there are two MHb reductases. It is conceivable that the CO could block the binding of TPNH to the enzyme and not effect the binding of DPNH but if one compares the MHb

reductase to other known reductase enzyme sequences, the point of attack generally attributed to CO would fall after the binding of the pyridine nucleotide in the reaction sequence. Hence, it is unlikely that the latter explanation is the reason for the CO sensitivity exhibited by MHb reductase T, and the explanation left for the differential effect of CO is that there are two MHb reductases.

The sensitivity of the MHb reductase T to CO adds confirmation to the work of Kiese (13) and of Heunnekins and co-workers (12) who claimed to have isolated a heme containing MHb reductase. The absolute requirement of their enzyme for methylene blue has in the past cast some doubt on the validity of their claims. Several workers (4,8) have used CO uptake to follow the rate of MHb reduction in whole cells. The reduction, under these conditions, can only be due to the presence of an MHb reductase D, providing further evidence in support of the hypothesis that two reductase enzymes are present.

The rates of MHb reduction under the various conditions in this investigation form an interesting pattern when compared to each other, and to the metabolic data available for the erythrocyte. Of the various reduction rates, the slowest is that found in the whole cell, while the greatest is that found in the lyophilizates when the purified pyridine nucleotides are used as the substrates. The rates with glucose-6-P and triose-P were in between these two extremes. This order of activity is that which would be expected from the metabolic data available.

TABLE XVII

Rates of MHb Reduction in Various

Enzyme Preparations

Enzyme Preparation	Substrate	Initial Reduction Rate µmoles MHb/min.
Whole cells	glucose*	0.00074
Hemolysate		
Procedure I	Glucose-6-P	0.0043
	Triose-P	0.0053
Procedure II	TPNH	0.0170
	DPNH	0.0091, 0.011

* These figures were calculated for an initial MHb concentration of 70%.

All data are based on an Hb concentration of 10 g %.

According to Denstedt and Brownstone (44), the rate limiting factor in the metabolism of the intact cell is the hexokinase activity. As a result, the rate of MHb reduction in the whole cell would be expected to be the lowest. The velocity, when purified pyridine nucleotides were used, would be considerably faster and the rate with metabolic substrates would fall in between. The results obtained agree with these concepts.

The activities of the various metabolic pathways limit the rate of reduction by the MHb reductase that is dependent upon them for reduced pyridine nucleotide. In the erythrocyte, the amount of DPNH made available over any given interval is 9 times the amount of TPN^+ reduced, as the glycolytic sequence is much faster than the pentose phosphate pathway (10). In cells, the di and triphosphopyridine nucleotides are found as DPN^+ and TPNH (43) partly because of the Redox potential of these two and partly because of the roles they play in the overall metabolism. These opposing

concepts, coupled with the fact that the MHb reductase T is faster than the D enzyme, form the basis for interesting speculations.

It is a natural conclusion that, during periods of methemoglobinemic stress, the erythrocyte should use all its facilities to return to the normal state. The data above also suggest that, in the normal cell, the status quo is maintained by the MHb reductase T. There will be a ready supply of TPNH and the MHb reductase T has a faster rate, so the probability of any given MHb molecule being reduced by the MHb reductase T would seem to be higher than the probability that MHb reductase D would perform the same reduction. Further evidence for this, comes from the work of Gibson (8) who found a steady level of pyruvate in the normal cell, which means that the DPNH produced is normally utilized for the reduction of pyruvate to lactate and is not available for the reduction of MHb. Under methemoglobinemic stress, pyruvate accumulates, suggesting that the normal processes are overwhelmed and the DPNH is being tapped for the reduction of MHb. Of course, the statement that the normal cell maintains the Hb via the MHb reductase T is speculation. As was pointed out earlier, the metabolism of the normal and methemoglobinemic cells has not been sufficiently studied to allow conclusions to be drawn on this topic.

The activation of MHb reductase D by EDTA poses questions that cannot be answered at this time. EDTA is a very non-specific chelating agent, hence the ion that is being sequestered cannot be easily identified. The addition of ions to the reaction mixture will only increase the amount of EDTA required to prevent the inhibition. If it is to be identified, the inhibiting ion must be removed during the preparation of the lyophilizate and only then will the addition of ions produce a true inhibition. The results eliminate Na^+ and K^+, as these are present in

amounts far greater than the amount of EDTA required to prevent inhibition.

A reductase enzyme has two substrates; an electron donor and an electron acceptor. In the case of MHb reductase D, the rate of the reaction is independent of the concentration of the donor substrate (DPNH) when our methods are used. The concentration of the donor and acceptor substrates are close (within a factor of 5) when the assays are being performed, and there are no apparent effects of DPNH concentration on the reaction rate until the DPNH concentration is about 1/100 the concentration of the MHb. Thus, the apparent association constant of the DPNH and the enzyme must be higher than the constant for the association of MHb and enzyme, and the DPNH must be bound more rapidly than the MHb. This could be because the enzyme is reduced itself before the binding of MHb, or because the DPNH-enzyme complex is the more stable complex. In either case, the DPNH would be bound first and then the MHb.

As the association constant of the DPNH is the higher of the two, the rate of the reduction will be controlled by the association of the enzyme and MHb or by the reduction itself. The question arises as to whether the active complex of the enzyme contains one or two MHb molecules. This comes from the fact that the donor substrate loses two electrons and the heme that is reduced receives only one. As previously mentioned (General Introduction), the various heme groups in Hb are separated by a minimum of 25A which makes the simultaneous transfer of electrons to 2 different heme groups on a single Hb molecule rather difficult. The first alternative would be to bind two MHb molecules and to reduce one heme on each molecule. The second alternative would be to have, somehow, a one electron transfer mechanism, which would mean the binding of only one MHb in the active complex of the enzyme mechanism.

The usual way that one determines the number of molecules bound in the transition state is to adjust the enzyme and substrate concentrations so that the rate is directly proportional to the substrate concentration (or a power thereof). Under the experimental conditions it was impossible to vary the concentration of the enzyme and MHb independently as the two are found in an inseparable mixture in the lyophilizate. The solution would seem to be, to find a relationship that would allow the enzyme and substrate to be varied simultaneously and still produce the required information.

In chemical reactions, when two species are involved in a reaction, the rate expression is (45):

$$V = K [A]^n [B]^n$$

However, enzymic reactions differ in that the activity does not vary directly with the substrate concentration (43), and the rate must be expressed as:

$$V = K [E] (f [S_1])^n$$

Under specific conditions, in which the concentrations of enzyme and substrate are in a constant ratio, this equation may be reduced to either:

$$V = K_1 [E]^{n+1} \qquad \text{or to:} \qquad V = K_2 (f[S_1])^{n+1}$$

The derivation of these equations, when both Michaelis-Menton and Steady State kinetics are involved, is to be found in the Appendix III.

Under experimental conditions, the enzyme concentration is seldom in moles and some other units are employed (usually grams of protein). The M concentration of the substrate is usually available but

but under our conditions the grams of protein represents both the enzyme concentration and the MHb concentration, and the above equation is equal to:

$$V = K_3 \text{ (g protein)}^{n+1}.$$

To determine the number of MHb molecules involved in the rate determining step one has to evaluate n from plots of V vs (g protein)^x. In the MHb reductase D system, $n = 1$ as the value of x when the graph is linear when $x = 2$.(fig. 9,10). Although the rate limiting step is dependent upon the first power of the MHb concentration, the overall mechanism may still require the binding of two MHb molecules, the first rapidly and the second much more slowly. In this case the kinetics of the reaction would normally show dependence on the MHb concentration to the first power, but when the reaction mixture is diluted, the binding of the first MHb molecule might become apparent in the kinetics as a shift of n towards 2. The statistical regression line (fig. 9,10) would be lowered to intercept the abscissa if the value of n were to show such a tendency. However, the opposite occurs, i.e., the regression line intercepts the ordinate, so the value of n must tend to 0 as the system is diluted. Because of this the probability that the mechanism involves the binding of two MHb molecules is less than would otherwise be the case.

Alberty (46) has summarized a variety of mechanisms that have been useful for interpreting kinetic data. Without considering these in detail, it may be stated that from the data presented in Table XV and fig. 8,9,10, that the mechanism of MHb reductase D is one of the following:

1) Mechanism involving two binary complexes:

$$E + DPNH \rightleftharpoons E \cdot DPNH$$

$$E \cdot DPNH \rightleftharpoons EH_2 + DPN^+$$

$$EH_2 + MHb^{4+} \rightleftharpoons E + MHb^{2+}$$

and then the MHb^{2+} re-enters another reaction to become Hb.

2) Mechanism involving a single ternary complex:

$$E + DPNH \rightleftharpoons E \cdot DPNH$$

$$E \cdot DPNH + MHb^{4+} \rightleftharpoons DPNH \cdot E \cdot MHb^{4+}$$

$$DPNH \cdot E \cdot MHb^{4+} \rightleftharpoons E + DPN^+ + MHb^{2+}$$

or the ternary complex may dissociate in two stages producing either of the two possible binary E - product complexes. Both of these mechanisms assume that the MHb reductase binds only one MHb molecule at a time, and that the MHb to Hb reduction occurs in two stages. There is no evidence to contradict the idea that the 4 hemes in a hemoglobin molecule are reduced at the same time. Indeed, it is not known whether a hemoglobin molecule will exist with one, two or three of the heme groups in the oxidized form. Because of our very scant knowledge of the oxidation of hemoglobin, the whole task of determining how the MHb is reduced is immeasurably complicated. Of the statements that have been made concerning the reduction mechanism, none are free of conditions and any further statements can only be pure speculation.

The inhibitor studies show one definite fact; that a sulfhydyl group is involved in the reduction. Whether this group is present in the MHb substrate or the enzyme cannot be stated. The average assay contains approximately 10^{-6} moles of Hb. Perutz and coworkers (47) report that horse Hb contains 2 thiol groups that are

available for reaction with PCMB in the native protein. Hb from other species are reported to have more (48).

Perutz et al (28) report the position of the thiol groups in horse Hb. The distances between the thiol groups and the hemes of Hb are such that the thiol could very well be instrumental in the binding of the Hb by the reductase enzyme.

There are two stages discernible in the effect of PCMB on the reduction rate. When 0.5×10^{-6} moles of PCMB had been added to the reaction mixture there was no inhibition of the enzyme (see Table XVI) but when 1.65×10^{-6} moles of PCMB were present the MHb reductase D was inhibited 45 per cent. Thus it would appear that 1 of the thiol groups in the system may be titrated without loss of reductase activity but when the second begins to react the activity is lost. Further additions of inhibitor were limited by the solubility of the PCMB.

The recent work of Benesch and Benesch (49) shows that the thiol groups of Hb are not directly involved in the Bohr effect and so are not likely to be directly involved in the transfer of electrons to the hemes during the reduction process. The PCMB has then two possible points of attack. It can attack either the enzyme or the Hb preventing the formation of the enzyme-MHb complex. If the attack is on the MHb molecule, the differential effect of titrating the first thiol is easily explained. The tertiary structure of the MHb molecule permits two sites for the attachment of the enzyme, hence, the titration of one of the thiol groups would only block part of the substrate binding sites. When titration of the thiol groups proceeds to the point where 2 thiol groups are reacting inhibition of

the reductase is marked. Only speculations are available to explain the effect of PCMB if the thiol involved is part of the enzyme structure.

Quinine and Atabrine are known to inhibit flavo-enzymes. However, Heunnekins (50) could not find any protein bound flavin in the erythrocyte. Kiese has retracted his statements concerning the presence of flavins in MHb reductase T. It is possible that the inhibition is due to an interaction between the anti-malarials and some chemical entity in the MHb reductase that has a similar chemical nature to flavin (e.g. quiniod).

Two pressing questions are left unanswered by the study. Why is the reductase activity lost when the cell is destroyed by methods other than freeze drying, and why must the lyophilizate be diluted with saline to preserve the MHb reductase activity of the solution? It is possible that some irreversible dissociation occurs when the ionic strength of the medium is decreased, or that some reaction occurs when the cell is destroyed by other means than the one employed and in turn, causes the destruction of the MHb reductase. It is known that the MHb reductases are protected if the cell solution is fortified with nicotinamide before the cells are hemolysed (18). Something happens to the MHb reductases when erythrocytes are hemolysed but just what happens is unknown.

THE ERYTHROCYTE DYE REDUCTASE

I. Introduction

The erythrocyte is an aparticulate cell and, therefore, has no aerobic metabolism. Also, there can be no protein synthesis and only negligible synthesis of lipids and other materials. In view of these facts, the presence of a very active dye reductase (diaphorase) is surprising. Diaphorase, as it was first isolated by Straub (52), came from the cell particles. The theories and speculation prior to 1959 placed it among those enzymes concerned with aerobic metabolism. Recently, Massey (53) showed that the Straub disphorase was lipoyl dehydrogenase, an enzyme of the citric acid cycle, and concerned with the formation of acetyl-CoA. Neither of these sequences in which diaphorase is utilized is found in the erythrocyte, but the diaphorase or dye reductase activity is present.

The dye reductase activity may be explained in two ways. The enzyme may be vestigial, left over from the period of erythropoesis, or it may be a side reaction of some other enzyme normally present in the cell. In the literature, the MHb reductases have been theoretically joined with dye reductase activity (8), and some indirect evidence (15) has appeared as was mentioned in the general introduction. Blood dye reductase or diaphorase has never been isolated and there have no studies (other than 15) of this enzyme reported in the literature.

Since the isolation of the MHb reductase is one of the goals of these studies, and as the dye reductase was allegedly a MHb reductase,

a study of the dye reductase paralleling our investigation of MHb reductase seemed in order. In addition, the assay method could be used to estimate the dye reductase activity of various steps in a MHb reductase purification procedure to show whether the two activities did indeed come from a single protein.

II. The Dye Reductase Assay

A. The Method

The method described by Mahler for diaphorase (54) was modified for the study of the blood enzyme. These modifications consisted of a different order of mixing the ingredients, and a much higher dye concentration. In the assay, each cuvette contained, in a 3 ml volume: 100 μmoles of either Tris or Phosphate buffer, pH 7.6, 0.25 μmoles of DPNH, 0.2 ml of dilute hemolysate solution (1:25), and sufficient of the dichlorophenol indophenol solution described by Savage (55) to produce an optical density of approximately 3.0 when the preliminary reductase (vide infra) reaction had gone to completion. The dye, buffer, enzyme, and water were mixed, in the cuvette, placed in the spectrophotometer and left until the recorder indicated that the preliminary reaction was essentially complete. Then the DPNH was rapidly mixed in and the true dye reductase activity recorded as the change in E_{600} of the dye as the reduction proceeded. The recorder tracings were converted to readings in the log of optical density and plotted to obtain a rate graph. The slope of the line obtained was taken as the reaction constant (K^1). Correction for the chemical reaction between the dye and substrate was applied to give the corrected rate constant, K_c^1.

B. Corrections and Interfering Reactions

1) Chemical Reaction between the Dye and the Substrate

The diaphorase rate must be corrected for the slow chemical reaction between the dye and the substrate, DPNH. In Mahler's method the chemical reaction rate was determined prior to the addition of enzyme, but with the blood dye reductase, this procedure results in the measurement of the preliminary dehydrogenase reaction instead of the true enzyme rate. Therefore, the chemical reaction was determined in a separate cuvette, containing all but the enzyme solution and with the same dye concentration as was present when the reaction was started in the enzymic assay. The chemical rate was substracted from the enzymic rate so that:

$$K^1 - K^{chem.} = K^1_{corrected}$$

and K^1_c was taken as the true rate constant.

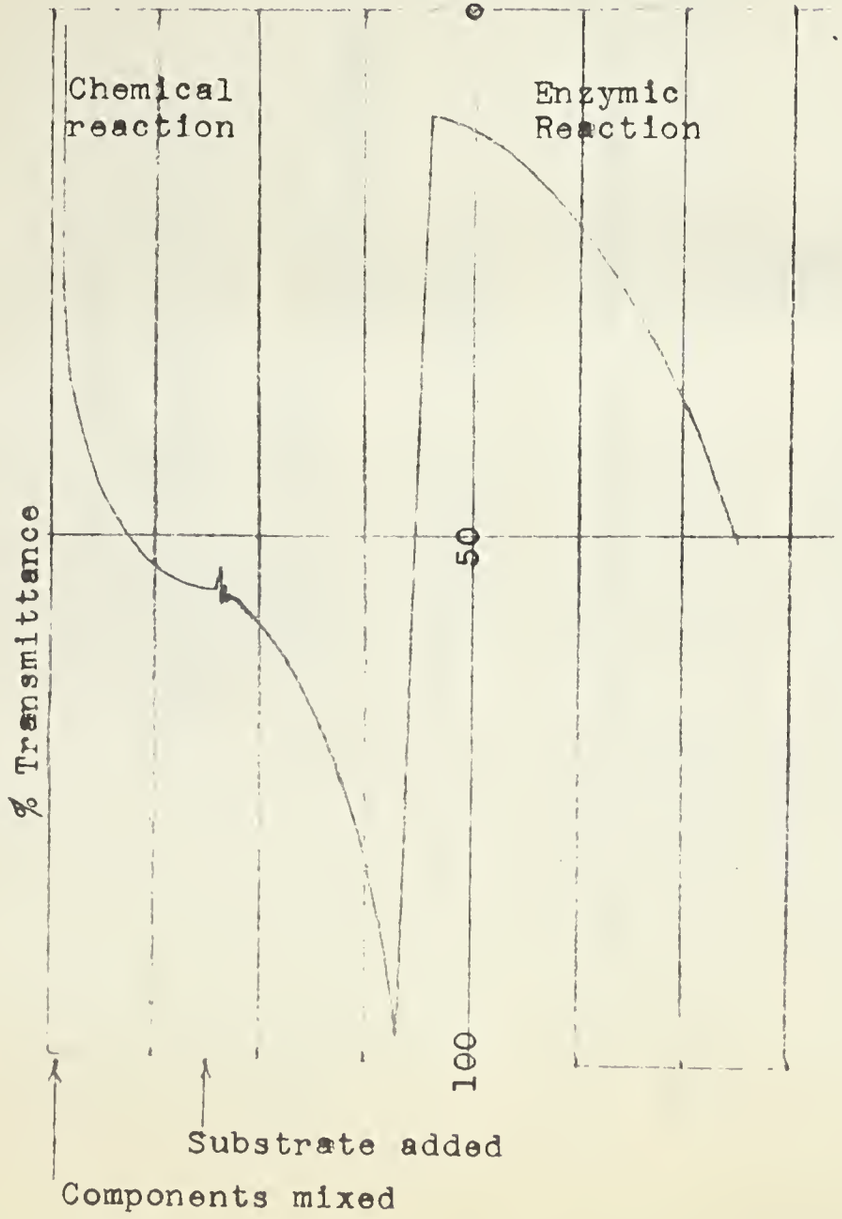
2. The Preliminary Reaction

When the hemolysate solution and the dye were mixed, even in the absence of DPNH, the dye was reduced. The reduction was sensitive to quinine and Atabrine, and was totally eliminated by the presence of PCMB. Zimmerman and Pearse (56) labled this type of spurious activity "Nothing Dehydrogenase". Correction for this false activity, by the same procedure used for the chemical reaction, is difficult as the rate varies somewhat with the state of the Hb - MHb equilibrium and the rate order of the spurious activity is not the same as that of the dye reductase itself.

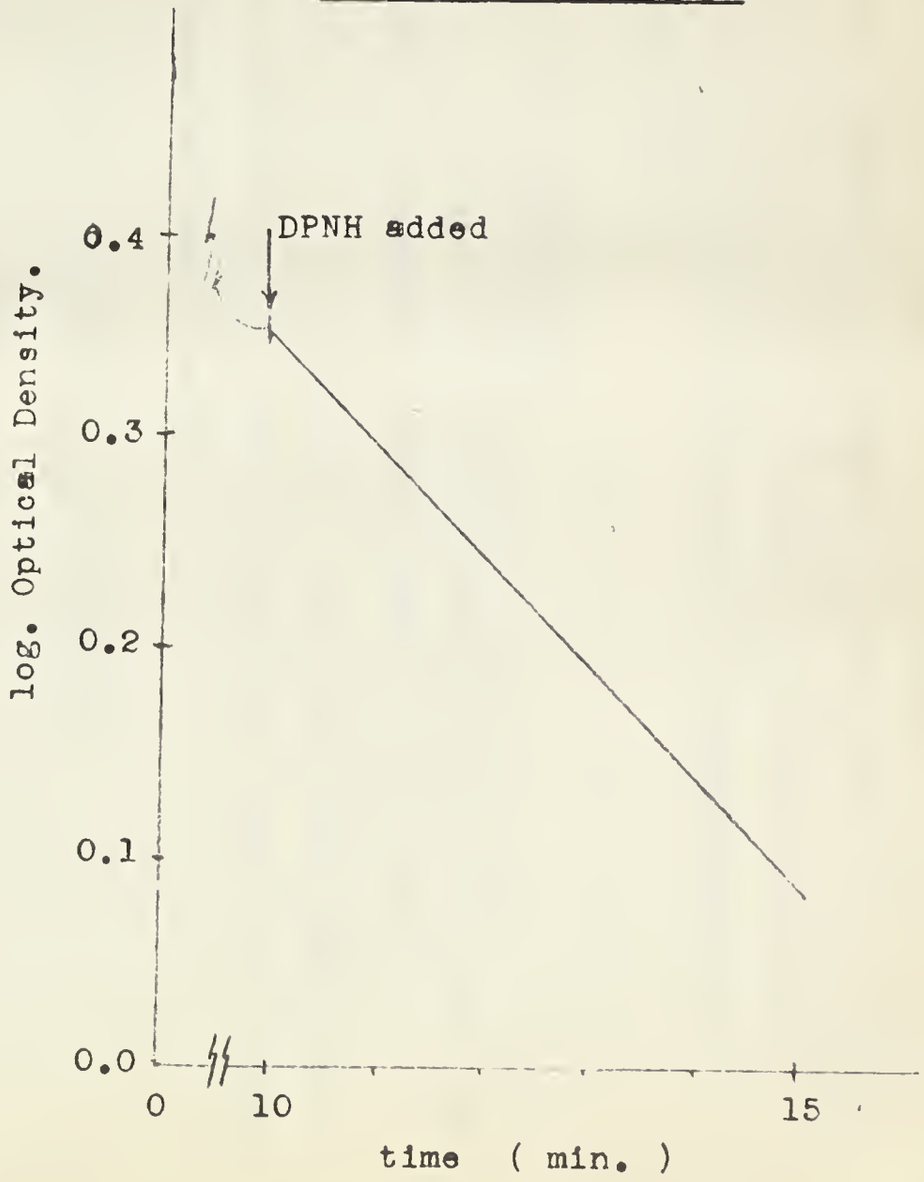
A recorder tracing of a typical assay is shown in fig. 11. The preliminary dehydrogenase reaction was soon finished and the loss in time incurred while this reaction proceeds, may be offset by mixing the

Fig. 11

A Typical Recorder Tracing



A Typical Rate Graph



reagents during the previous assay and allowing the curvette to stand during the time that the previous assay is being run. The dye reductase reaction was started when the preliminary reaction was essentially complete, and no further correction for the "nothing dehydrogenase" activity was made.

C. The Instrument

The recording spectrophotometer used in these studies was assembled from a variety of commercial units. The instrument is described in detail in the Appendix. The design allowed readings from 0.00 - 4.00 optical density units to be read from a meter or recorded on a time-drive strip chart recorder.

III. The Rate and Order of the Dye Reduction

A. Theoretical

The rate may be expressed as a specific rate function:

$$- \frac{dC_{dye}}{dt} = K\, C_{dye} C_{DPNH}$$

C = concentration of reactant t = time K = specific rate constant. When the DPNH concentration is above 50 μM a pseudo-first order rate results:

$$- \frac{d\ C_{dye}}{dt} = K^1\ C_{dye}$$

which on integration becomes:

$$K^1\, t = 2.303 \log \frac{C_o - C}{C_o}$$

C_o is the original dye concentration

C is the concentration at time t.

The measure of $C_o - C/C_o$ is the change in E_{600} of the dye during reduction. The characteristic of this type of reaction rate is that the rate constant (K^1) is independent of the initial concentration of the rate limiting material. Within the range of the instrument the rate order could not be reduced to zero.

B. Experimental

1. Independence of DPNH and Dye Concentration

The reaction rate was constant within a wide range of concentrations of both the electron acceptor and donor substrates. The initial concentration of the dye is not critical because of the reaction order being first with respect to the dye, and the DPNH concentration was maintained above the critical 50 μM level. Sufficient DPNH was added to each assay cuvette to ensure that the reaction would proceed for 2-3 min. without a change in the reaction kinetics.

2) The Effect of Enzyme Concentration

At low concentrations of enzyme solution, the reaction rate varies directly with the amount of protein added to the system. As the protein concentration in the cuvette is increased, the rates of endogenous substrate reductions cannot be ignored. The net effect of these outside influences is an upward curve in the enzyme concentration vs. rate graph (fig. 12). At higher protein concentrations the dye reductase activity falls off, possibly due to interactions between the protein molecules or because of limitations of the method imposed when the heme concentration becomes very high and the instrument cannot measure the changes that are occurring. To minimize these effects the assays were

TABLE XVIII

Effect of Dye Concentration on the Rate

of Erythrocyte Dye Reductase

Dye Concentration (final M x 10^5)	K_c^1 min. $^{-1}$
15.0	0.089
16.1	0.090
16.6	0.088
18.7	0.091
20.7	0.090
22.9	0.089
25.0	0.089
30.0	0.090

TABLE XIX

DPNH Concentration and the Rate of

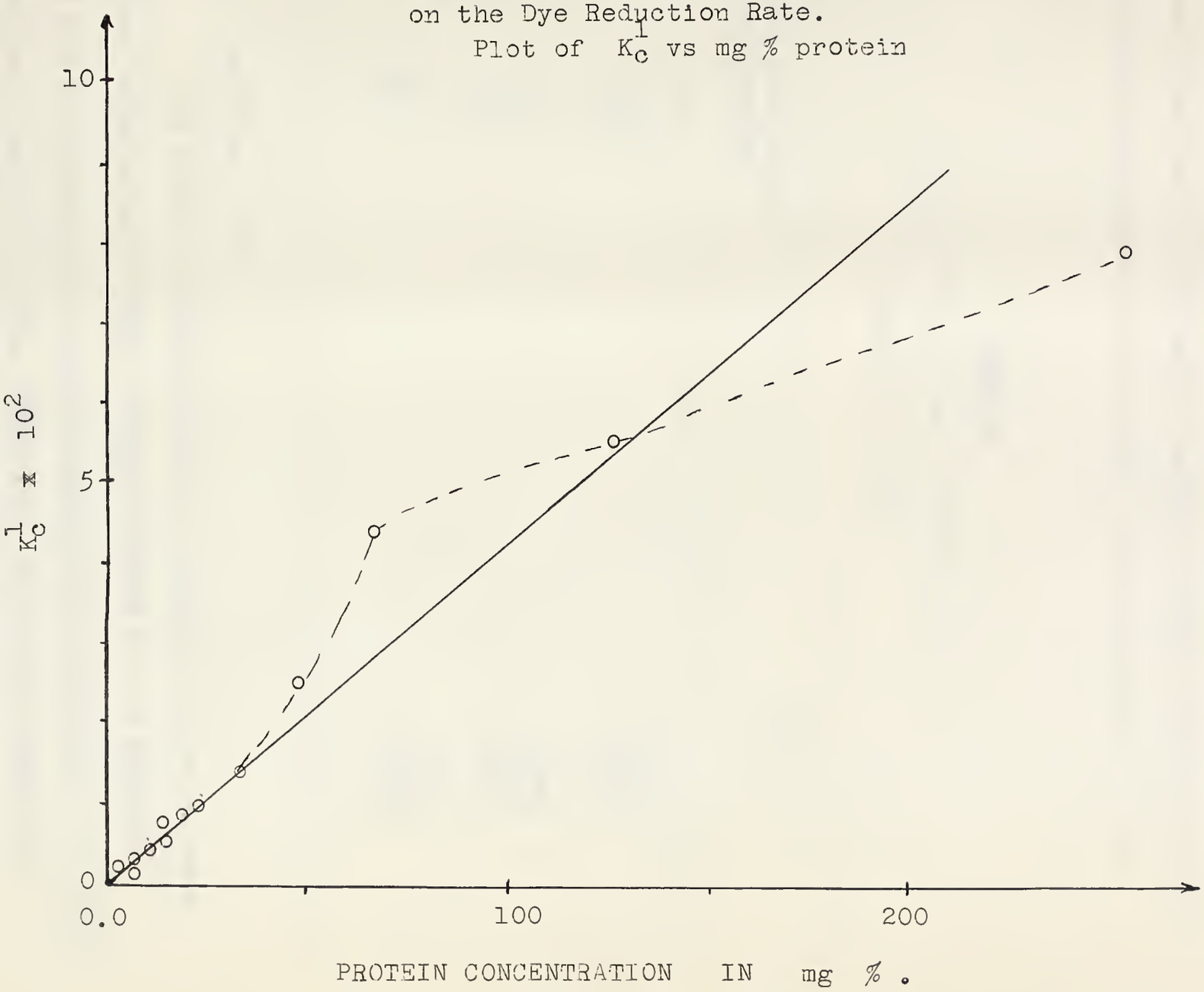
Erythrocyte Dye Reductase

DPNH Concentration (final M x 10^3)	K_c^1 min. $^{-1}$
0.048	0.090
0.064	0.088
0.080	0.081
0.096	0.084
0.112	0.082
0.128	0.077
0.144	0.079
0.160	0.084

Fig. 12

The Effect of Enzyme Concentration on the Dye Reduction Rate.

Plot of K_c^1 vs mg % protein



performed with a protein concentration below 50 mg per cent.

The dye reductase activity of a solution of 1 mg per cent protein was, as a K_c^1 , 0.00047 $\pm$ 0.00009 min^{-1} at a pH of 7.5. This figure is the result of 40 assays on several different enzyme preparations. The protein concentration in these assays was between 10 and 70 mg per cent.

TABLE XX

Protein Concentration in Relation to

Dye Reductase Activity

Protein Concentration in mg per cent	K_c^1 min. $^{-1}$
5.09	0.00275
10.2	0.0045
12.7	0.0059
20.4	0.0086
25.4	0.0120
50.8	0.0245
63.5	0.0432
127.	0.0548
254.	0.0787

IV. The Effect of pH

The normal bell-shaped pH curve of enzymes could not be demonstrated using our assay method. The dye involved is a pH indicator, pK_a near 5.6. The E_{600} is a characteristic of the ionized salt. Since the reaction is always first order, and because the pH curve follows the

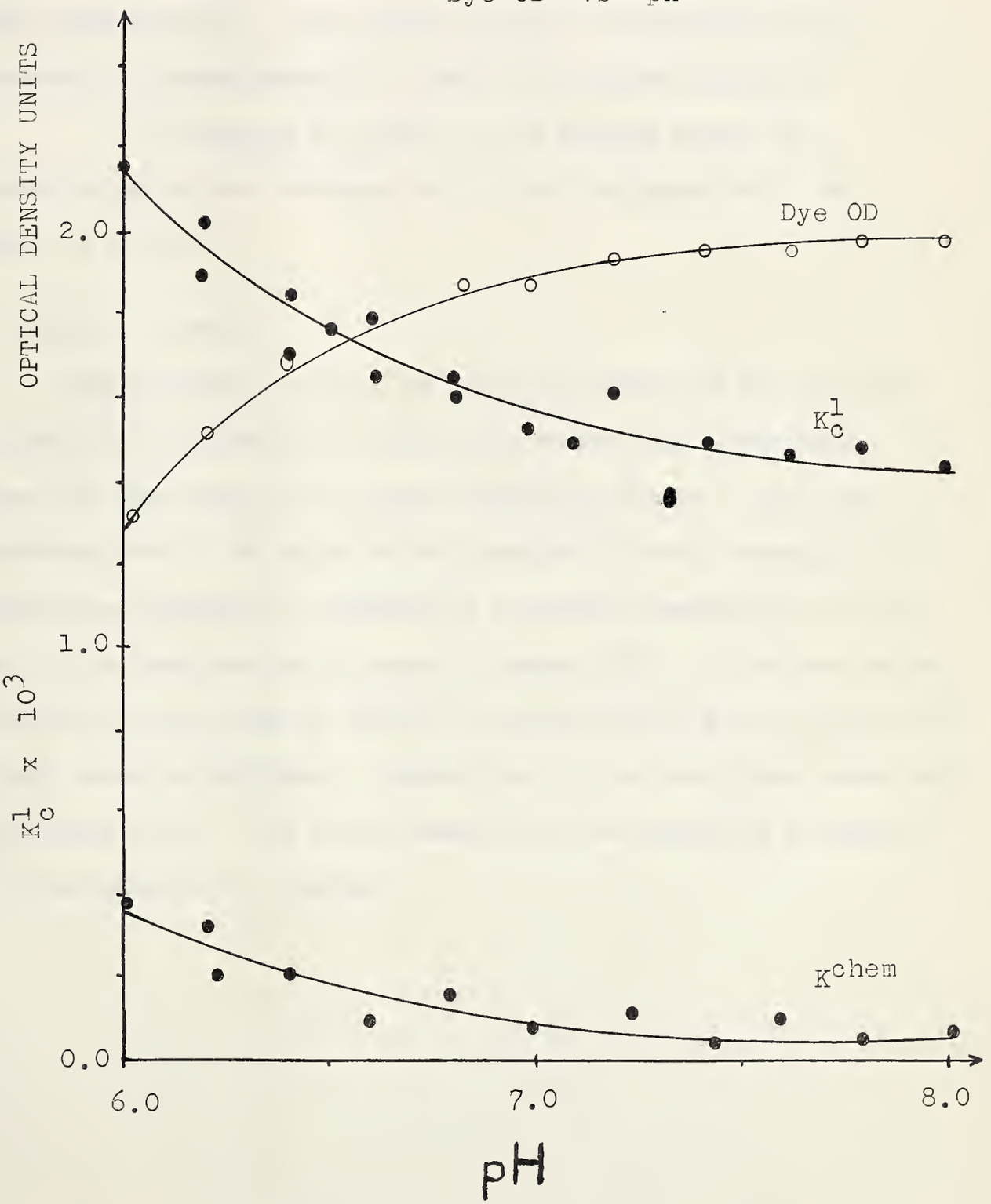
Fig. 13

The Effect of pH on Dye Reductase

Plots of K_c^1 vs pH

K^{chem} vs pH

Dye OD vs pH



titration curve of the dye, the enzyme must bind the unionized phenol. The optimal pH would be one where the dichlorophenol indophenol would not be ionized and so would not exhibit the 600 mu absorbtion required for the assay. Fig. 13 shows the pH curve of the enzyme activity as measured and the titration curve calculated for the dye. The two have similar characteristics. The broken portion of the enzyme curve is theoretically extended beyond the limits of the assay procedure.

To minimize the effects of pH changes during the reaction period the dye reductase activity was estimated with a pH between 7.4 and 7.6.

V. Inhibitor Studies

Dye reductase activity has been associated with flavoproteins since the first diaphorase was isolated by Straub (52). Many flavo-enzymes have been shown to have some diaphorase activity. The crude preparations used in the study of dye reductase activity generally have contained heme enzymes that oxidize the leuco dye, resulting in serious errors if the hemes were not blocked by cyanide (55). If the erythrocyte enzyme were the same type of enzyme, the anti-malarial drugs, quinine and Atabrine, would be inhibitory. Cyanide ion, on the other hand, would have a stimulatory effect. The blood reductase was not sensitive to either of the anti-malarials or to cyanide.

Fig. 14

Inhibition of Erythrocyte Dye Reductase
by p-chloromercuribenzoate

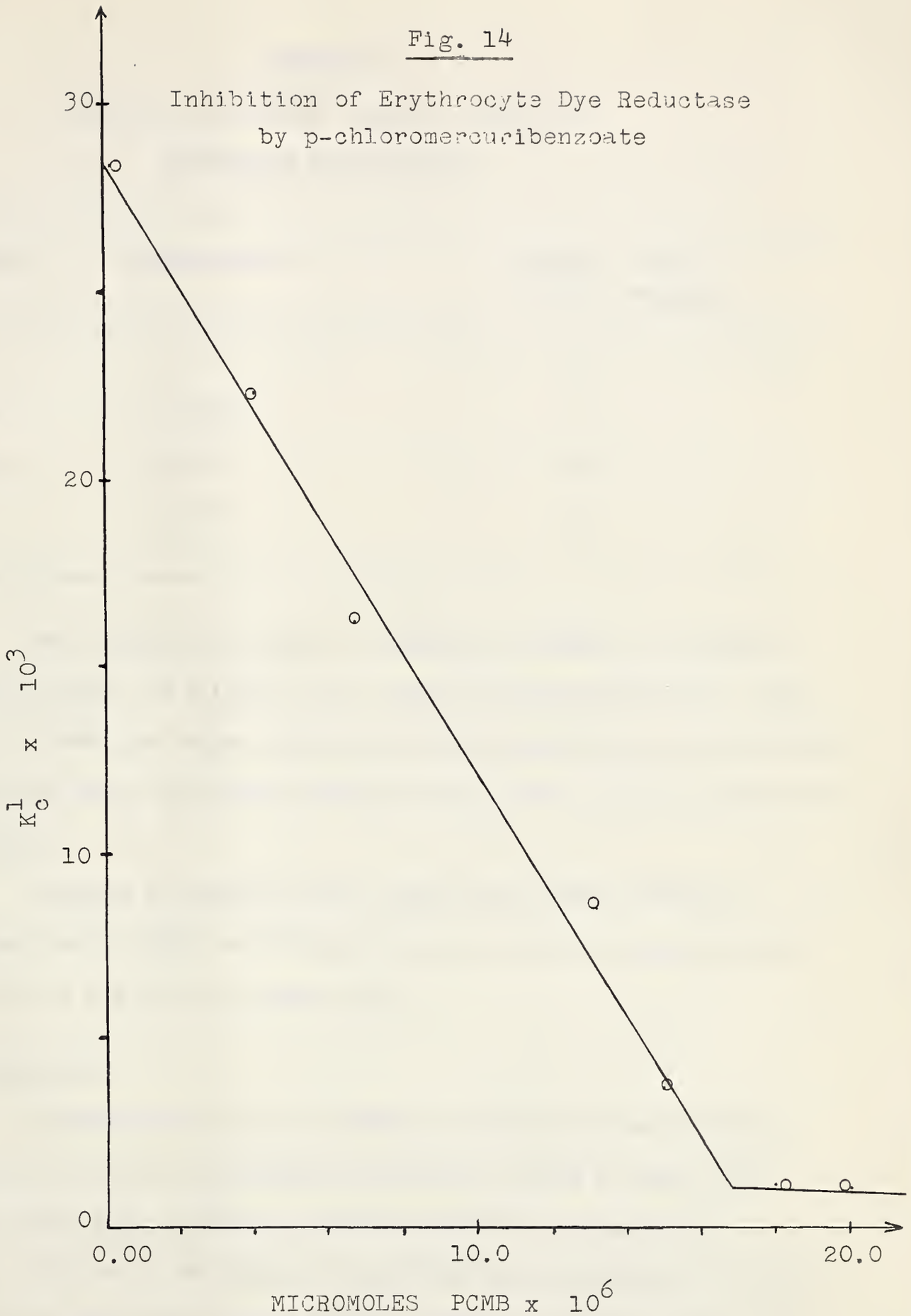


TABLE XXI

The Effect of Quinine, Atabrine and CN^- on

Erythrocyte Dye Reductase

Inhibitor	Concentration	K_c^1 min^{-1} x 10^4 (per mg % protein)
None	-	4.7
Quinine	1×10^{-3}	4.9
Atabrine	1×10^{-3}	4.5
CN^-	1×10^{-4}	4.7

The interaction between dye reductase and PCMB, a sulfhydryl coupling reagent, was followed over a range of PCMB concentrations. The extent of inhibition varied directly with the concentration of the inhibitor and with the amount of protein present in the system. Fig. 14 illustrates this effect.

Attempts to duplicate these results with other sulfhydryl inhibitors such as Hg^{++} and Ag^+ failed because the Hb was precipitated by these ions at the pH of the enzyme assay.

VI. Discussion

A flavoprotein with dye reductase activity has been found in almost every tissue of the mammalian body and in those of many other animal species (54), generally associated with the cell particles. Possibly, because of the absence of particles in the erythrocyte, the dye reductase of this cell has been ignored. The only reported study of this

enzyme (15) appeared as a preliminary note and the final paper has not appeared.

Study of the erythrocyte dye reductase was greatly hindered by the preliminary dehydrogenase activity ("nothing dehydrogenase"). This spurious activity had many interesting and baffling aspects some of which are discussed by Keleti and Telegdi (58). Changing the order of mixing the assay components resulted in a technique that was essentially free of interfering reactions.

The reactive form of the dye substrate is the un-ionized phenol. The ionization equilibrium maintains an almost constant concentration of the substrate so the order of the reaction does not change until more than 70 per cent of the dye has been reduced. Since the active form of the dye is the un-ionized phenol, the concentration of DPNH may be very low before it comes close to that of the second substrate. The kinetics of the reaction are therefore free of a term for the concentration of DPNH except at extreme dilution of this substrate. The independence of the substrate concentration makes the dye reductase assay a very simple procedure as there are no accurate measurements required in the preparation of the reaction mixture.

The enzyme concentration in the dye reductase assay must be low. In an undiluted hemolysate, reduction of a dye proceeds instantaneously even when dye present is 5 or 6 times the concentration that is found in the reaction mixture. In addition, some factor increases the apparent dye reductase activity when the protein concentration in the solution becomes greater than 50 mg per cent. This effect could be an "overcorrection for the blank" caused by the oxidation of Hb in one of the cuvettes,

or it could be the presence of a dissociable cofactor or activator (57). However, most likely it is due to the recycling of the "nothing dehydrogenase" when the DPNH was present. Such a chemical reaction would be slow compared to the preliminary reaction hence is normally diluted to inactivity in the dye reductase assay and becomes apparent at high protein concentrations.

The effect of PCMB upon the dye reductase activity indicates that the enzyme active site contains a thiol group. Although dye reductase activity is normally associated with flavo-enzymes, the inhibitors quinine and Atabrine had no effect on the enzyme. Cyanide does not inhibit the Straub (52) diaphorase but is added to the reactions to prevent the auto-oxidation of the leuco dye by the hemes that are present in crude enzyme extracts. If one assumes that in the preliminary reaction the dye and the Hb equilibrate, then the small amount of leuco dye produced in the enzymic reaction will not seriously change the equilibrium. Thus the auto-oxidation of the leuco dye in the critical first minutes of the enzyme reaction will be negligible, and the addition of cyanide ion will have no effect on the reaction rate. The experimental results confirm these ideas.

Insensitivity of the dye reductase to quinine and Atabrine inhibition is a point against the theory that MHb reductase D and dye reductase are the same enzyme as the MHb reductase is inhibited by these drugs. However, the apparent complexity of the MHb reductase action could permit the binding of the dye in such a way that the part of the enzyme that is affected by the inhibitor quinine was not involved in the dye reaction. The present data will not permit a definite statement to be made in this regard. This must wait for the isolation of the MHb reductases.

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APPENDIX

I. Preparation of Substrate Solutions

A. The MHb Reductase D, Substrate Solution, Procedure I.

The ADP and triose-P were obtained as the Barium salts. Before these compounds could be used the Barium had to be removed. Precipitation as $BaSO_4$ was not feasible as any excess sulfate would possibly inhibit the reductases (12).

The required amounts of ADP and triose-P were weighed by difference. The materials were dissolved in 1/4 the final volume of demineralized water. One ml of M K_2HPO_4 was added and the precipitate of Barium Phosphate centrifuged out. The supernatant was decanted. The remaining cofactors were weighed out, and dissolved in the supernatant. The solution was brought to the required volume with reaction buffer. If necessary, the solution was again centrifuged. One ml aliquots were used in the assay as the substrate solution.

The acid group on the substrate molecules were effectively neutralized by the K_2HPO_4. The pH of this substrate solution was around 7.0 - 7.2.

II. The Spectrophotometer Used in the Investigation

The spectrophotometer was built from a variety of commercial units. A Beckman D.U., vintage 1949, was modified as follows:

1) The photocells were removed and the housing modified to mount the photo-tubes of a Photovolt 520-M photomultiplier.

2) The Beckman circuits were disconnected.

3) A Beckman micro attachment was fitted.

4) Thermospacers were installed.

5) The old lamp housings were discarded in favour of a Beckman 6871 Dual Source Unit.

Voltage stabilized power for the lamps was obtained from a Beckman Model B Hydrogen Lamp Power Supply and a Nylab Voltage Stabilizer (Cat. No. 80710) (Tungsten lamp).

A Varian G-11A linear strip chart recorder was connected in series with the photomultiplier meter. This recorder required a shunt resistance of about 150 ohms (56 ohms fixed resistance and 200 ohms variable resistance).

The Photovolt 520-M unit had had its meter changed to a Bach-Simpson No. 9, 100 μampere meter so that the meter read directly in per cent Transmittance. The photomultiplier unit has an attenuator that was calibrated so that full scale deflections of 0 - 100, 0 - 10, 0 - 1, and 0 - 0.1 % Transmittance could be obtained.

With the recorder in operation slight deviations from linearity were noted in the regions of 0 - 10 and 95 - 100 per cent Transmittance (0 - 100 range) however, as the work was carried out between these points the error was ignored.

III. Derivation of Equations

The relationship between the velocity of an enzymic reaction and the concentration of enzyme and substrate when E/S = K.:*

$$V = K [E]^{n+1}$$

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* The above symbols are: V = reaction velocity; K_a = constants; E = enzyme; S_a = substrates; n = power of the term; p = the changing parameter; T = temperature; t = time; z = the number of cofactors.

Derivations:

1) The Treatment for Michelis Kinetics.

The Michelis theory states that the reaction depends on the formation of an enzyme-substrate complex, and the rate is directly proportional to the rate of formation of the complex (43). The equation that governs the association of the enzyme and substrate in the formation of the complex is:

$$[E \cdot_n S] = K [E] [S]^n \qquad (1)$$

When the enzyme and substrate follow the relationship;

$$[E] = K_1 [S] \qquad (2)$$

the equation (1) becomes:

$$[E \cdot_n S] = K [E] \left([E] / K_1 \right)^n$$

which reduces to:

$$[E \cdot_n S] = K_2 [E]^{n+1} \qquad (3)$$

Because the rate of formation of $[E \cdot_n S]$ determins the rate of the reaction:

$$V = K_2 [E]^{n+1} \qquad (4)$$

In terms of the experimental data of the MHb reductase assay (4) becomes:

$$V = K_3 (\text{g protein})^{n+1} \qquad (5)$$

2) The Treatment of the General Case. (Steady state kinetics)

The true rate expression of an enzymic reduction would be:

$$V = \left(\frac{\partial p}{\partial t} \right)_T = K [E] (f [S_1])^n (f [S_2])^n (f(\text{pH}^+)) (f [\text{cofactors}])^Z \qquad (a)$$

Under normal conditions, in which the pH and the cofactors become constants, a specific rate expression represents the rate:

$$V = \left(\frac{\partial p}{\partial t} \right) = K_o [E] (f [S_1])^n (f [S_2])^n \qquad (b)$$

When V can be obtained, independent of the concentration of one S, a new specific rate expression results:

$$\left(\frac{\partial p}{\partial t}\right) = K_1 [E] (f [S_1])^n \qquad (c)$$

When the experimental conditions are carefully controlled, the rate order may be reduced to,(d):

$$\left(\frac{\partial p}{\partial t}\right)_{S_1} = K_2 [E] \qquad (d)$$

For any given E, a hyperbolic expression for the effect of the concentration of S may be obtained,(e):

$$\left(\frac{\partial p}{\partial t}\right)_{E} = (f [S])^n \qquad (e)$$

When the function (c) is plotted on space coordinates (fig. 15), a surface results. This surface describes the relationship between V and the concentrations of E and S. The XZ plane carries the function (d) and the YZ plane, the function (e).

In the system under discussion, the condition that (f) holds has been arbitrarily imposed.

$$[E] = K_3 (f [S_1]) \qquad (f)$$

Equation (f) describes a line on the XY plane, and on the space coordinates, a plane, perpindicular to the XY plane. The required function is the line of intersection of the plane described by (f) and the surface that represents (c).

By solving (f) for (f(S)) and substituting in (c) we get:

$$\left(\frac{\partial p}{\partial t}\right)_{ES} = K_1 [E] ([E]/K_3)^n \qquad (h)$$

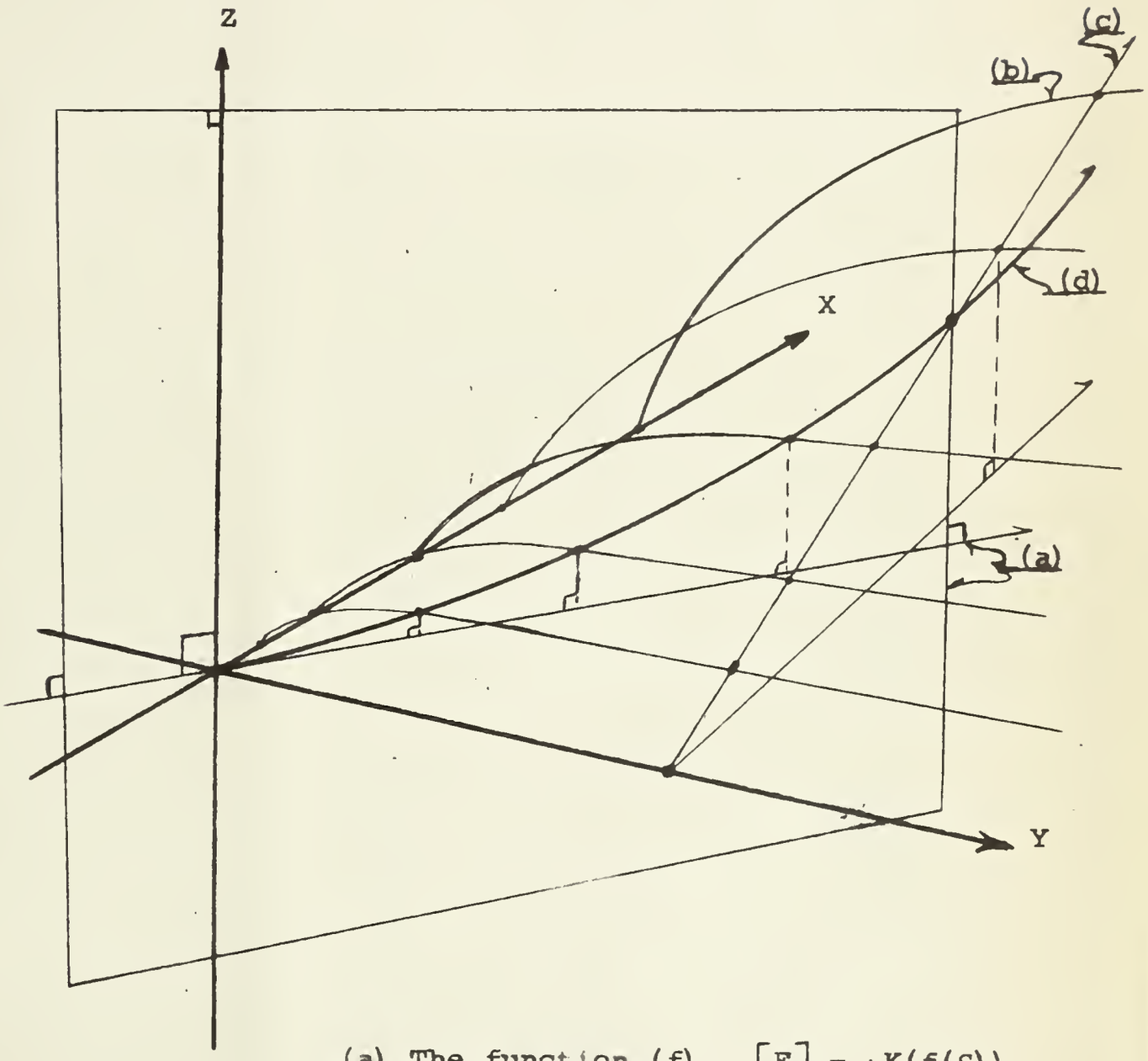
which, when K_1 and $(1/K_3)^n$ are combined in K_4, becomes:

$$V = \left(\frac{\partial p}{\partial t}\right)_{ES} = K_4 [E]^{n+1} \qquad (i)$$

which is the required function.

Fig. 15

A Graph on Space Coordinates
Representing the Equation
$V = K\left[E\right]^{n+1}$



(a) The function (f) $\left[E\right] = K(f(S))$
(b) The function $f(S)$
(c) The function $V = K\left[E\right]$

(d) The Required function $V = K\left[E\right]^{n+1}$

The line (a) is the named function in the XY plane and the plane shown is the representation of the same equation on the space coordinates.